

## Mutations in *Helicobacter pylori* *porD* and *oorD* Genes May Contribute to Furazolidone Resistance

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**Aim** To determine the rate of furazolidone resistance of *Helicobacter pylori* (*H. pylori*) isolated from gastric biopsy specimens and to explore the relationship between genetic mutations in *porD* and *oorD* genes of *H. pylori* and its resistance to the antibiotic.

**Methods** Gastric biopsy was performed in 83 adult patients aged 31-77 years with gastric complaints. *H. pylori* was isolated from biopsy specimens of 46 patients. E-test and 2-fold agar dilution method were used to determine the rate of *H. pylori* resistance to furazolidone. The genes *porD* and *oorD* from susceptible and resistant isolates were amplified by polymerase chain reaction (PCR), and their PCR products were sequenced.

**Results** Resistance to furazolidone was found in 8.7% of *H. pylori* isolates and 6 mutations were detected in *porD* and *oorD* genes of the resistant isolates. Three mutations – G353A, A356G, and C357T – occurred in *porD* and the other mutations – A041G, A122G, C349A(G) – occurred in *oorD* genes.

**Conclusions** Changes in 6 amino acids may be associated with the resistance of *H. pylori* to furazolidone.

*Helicobacter pylori* (*H. pylori*), a microaerophilic, gram-negative, spiral-shaped bacterium, infects the stomach of more than half of the human population worldwide (1). The prevalence of *H. pylori* infection greatly varies (2). Eighty percent of middle-aged adults are infected with *H. pylori* in developing countries, but only 20%-50% in developed countries (3). The organism has been accepted as the etiological agent of many digestive diseases (4). Clinical experience has demonstrated that *H. pylori* infection is not easy to cure. The primary obstacles to successful treatment are lack of patient compliance with the drug regimens and the development of antibiotic resistance (5).

Metronidazole, a prodrug that can sterilize the gastric epithelium by inducing *H. pylori* DNA strand breakage, helix destabilization, and unwinding (6), remains a major component of new triple and quadruple therapies for successful treatment for *H. pylori* infection (7). Monotherapy with metronidazole results in the development of metronidazole resistance, found in more than 50% of *H. pylori* isolates. Furthermore, metronidazole is also highly mutagenic, and its use may result in the generation of resistance to other clinically used antibiotics, such as clarithromycin (8).

The other prodrug, furazolidone, used for the treatment of *H. pylori* infection (9), can also induce the resistance in *H. pylori* but the mechanism of this resistance is still not clear. Since bioactivity of furazolidone is similar to that of metronidazole, Kwon et al (10) presumed that *por* and *oor* genetic mutations of *H. pylori* were involved in furazolidone resistance.

Our aim was to determine the resistance rate of *H. pylori* to furazolidone in isolates from gastric biopsy specimens taken from the patients from Zhenjiang, Jiangsu province of China, and establish the relationship between possible mutations in *H. pylori porD* and *oorD* genes and resistance to furazolidone.

## Patients and methods

### Patients

A total of 83 patients (48 men and 35 women) aged between 31 and 77 years, who lived in Zhenjiang, were enrolled in the study because of gastric complaints. All patients underwent upper gastrointestinal endoscopy and gastric biopsy. Three biopsy specimens were taken from the antrum of the gastric body or the duodenal mucosa and processed within 3 hours. Two of the samples were used for the rapid detection of urease activity and pathologic diagnosis, and another one was used for biopsy culture. Most patients (n=74) were diagnosed with gastric ulcer, 8 had gastritis, and one patient was diagnosed with gastric cancer. Of 83 patients, 68 had previously received eradication therapy with metronidazole and clarithromycin.

### Biopsy culture

Gastric biopsy samples including NCTC11637 were routinely cultured on Columbia blood agar plates (Oxoid Ltd, London, England) containing 5% fresh sheep blood under microaerobic conditions at 37° for 3 days. Strains were identified according to colony morphology, Gram stain, urease, catalase, oxidase, and biochemical properties (11).

**Table 1.** Polymerase chain reaction (PCR) primers used to amplify portions of genes of *H. pylori* isolated from gastric biopsy specimens from 46 patients

Primer pair	Encoded protein (gene)	Nucleotide sequences	PCR fragment size (bp)
POR-A POR-B	Pyruvate oxidoreductase ( <i>porD</i> )	5'-GCAAGAAGTCATTGACGC-3' 5'-GGGGTGATAGGATAGGCT-3'	592
OOR-A OOR-B	2-oxoglutarate oxidoreductase ( <i>oorD</i> )	5'-TTTAGCACAAAGGAGAATG-3' 5'-AACTTGCGTAATAGGAT-3'	458

### Minimal inhibitory concentration of furazolidone

The minimal inhibitory concentration (MIC) for isolated *H. pylori* strains was defined as the concentration of a drug needed to inhibit 50% of *H. pylori* growth and determined by 2-fold agar dilution and E-test. Agar dilution plates were prepared with Jeller-Hinton (MH) agar as the base medium. Sheep blood was added to the MH base medium at a concentration of 5%. Furazolidone solution was prepared in sterile distilled water and was added to 5% sheep blood-MH base medium to achieve serial concentration of 0.5-3.0 µg/mL of furazolidone per mL (12). Fresh *H. pylori* isolates (3 days culture) were prepared in saline to an optical density. With a Steers-type replicating device, 1-2 mL of the adjusted inoculum was delivered to the agar plates. All plates were incubated under microaerobic conditions for 3 days. The MIC was defined as the lowest concentration of furazolidone that completely inhibited the growth of the inoculum (13).

### Isolation of DNA

*H. pylori* genomic DNAs were isolated as described previously by Majewski and Goodwin (14). In brief, *H. pylori* was diluted in phosphate-buffered saline (PBS), vortexed, and centrifuged at 13000 × g for 5 minutes. The supernatant was discarded, and the pellet was resuspended in 100 µL of Tris-EDTA (EDTA) buffer (pH 8.0, 1 mmol/L Tris-HCl, and 0.1 mmol/L EDTA), 500 µL of guanidine thiocyanate, 0.5 mol/L EDTA, pH 8.0, and 10% sodium lauryl sulfate (GES). The suspension was incubated for 5 minutes at room temperature, then placed on ice for 2 minutes. The mixture was added into 250 µL of ammonium acetate (7.5 mol/L) and placed on ice for 5 minutes. Then, 650 µL of chloroform solution was added and the mixture vortexed. After centrifugation at 13 000 × g for 5-minute, the supernatant was transferred into a new tube (calculate volume), diluted in 0.54 × dimethyl-carbinol, vortexed, and left at room temperature for 5

minutes. Centrifugation at 13 000 × g for 5 minutes was repeated and the supernatant discarded. The pellet was washed twice with 500 mL of 70% alcohol. The DNA samples were stored at 4°C in 100-200 µL of Tris-EDTA buffer until use.

### Polymerase chain reaction amplification of *porD* and *oorD*

The *porD* and *oorD* genes were amplified by polymerase chain reaction (PCR) in a DNA thermal cycler. The amplified fragments of *porD* and *oorD* had 592 bp and 458 bp, respectively (Table 1). The PCR conditions consisted of 1 cycle at 95°C for 5 minutes followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 48°C (*porD*) or 44°C (*oorD*) for 45 s, and extension at 72°C for 1 minute. The final step was extension at 72°C for 10 minutes. The PCR products were purified and sequenced (Shanghai GeneCore BioTechnologies Co. Ltd, Shanghai, China). The sequences were analyzed with the Blast engine for the alignment of two given sequences (<http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi>) and ExPASy for the translation of a nucleotide (DNA/RNA) sequence to a protein sequence (<http://au.expasy.org/tools/dna.html>).

## Results

### Antimicrobial susceptibility

*H. pylori* was isolated from gastric biopsy specimens from 46 patients of 83 included in the study. Of 46 *H. pylori* isolates, 42 were suscep-

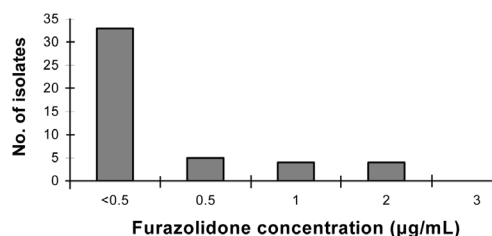
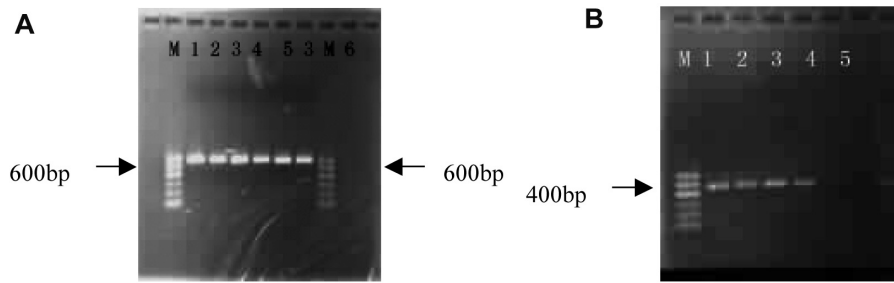


Figure 1. Minimum inhibitory concentration of furazolidone for *Helicobacter pylori* isolates from gastric biopsy specimens obtained from 46 patients.



**Figure 2.** Agarose gel electropherogram of *porD* (A) and *oorD* (B). A. Markers (from the left): NCTC11637, ZJ0021, ZJ0023, ZJ0027, ZJ0024, ZJ0021, and negative control. B. Markers (from the left): NCTC11637, ZJ0021, ZJ0024, ZJ0003, and negative control.

tible to furazolidone at MIC <2 µg/mL, whereas the remaining 4 were resistant to the drug at MIC >2 µg/mL. Furazolidone resistance was found in patients who had been treated previously with metronidazole. The similar results were found by using E-test. In addition, we found the resistance of 4 isolates at a low level of furazolidone (2-3 µg/mL) (Figure 1).

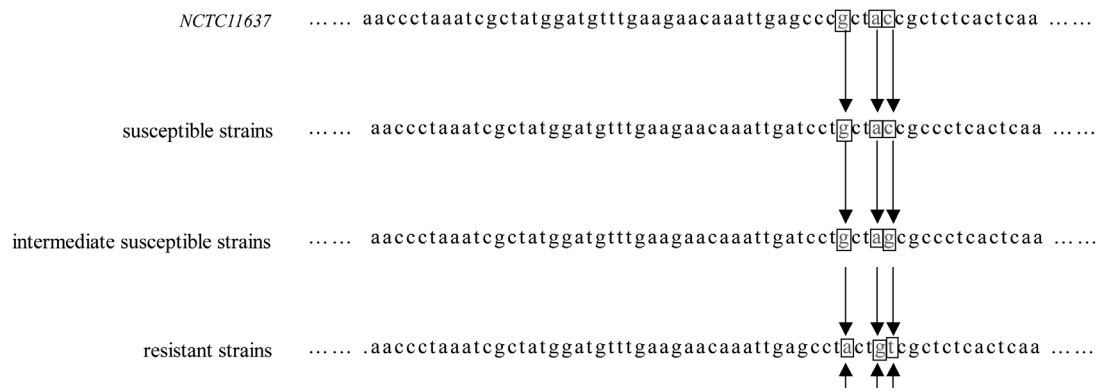
**Amplicons and sequence analysis**

The *porD* and *oorD* amplicons had 592 bp and 458 bp, respectively (Figure 2). By sequencing *porD* and *oorD*, we detected 6 amino acid changes in resistant isolates. Among these mutations, three (G353A, A356G, and C357T) occurred in *porD* gene and these mutations led to three amino acid changes; Glu→Asp, Ala→Thr, and Thr→Val, respectively (Figure 3). The other three – A041G, A122G, and C349A(G) – were found in *oorD* gene, causing three amino acid

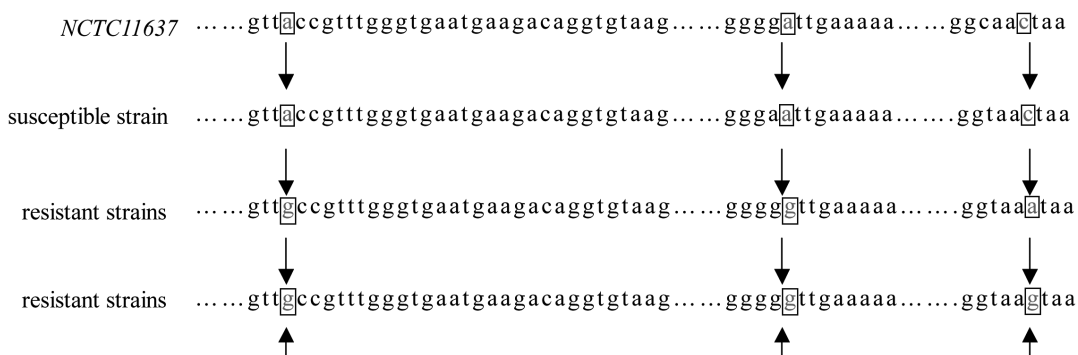
substitutions: Thr→Ala, Ile→Val and Asn→Lys, respectively (Figure 4). In addition, a synonymous substitution C357G at position 357 in *porD* was detected in intermediate susceptible strains to furazolidone (MIC = 1-2 µg/mL) (Figure 3). This substitution might suggest a transitional form of *H. pylori* from a susceptible one to that resistant to furazolidone.

**Discussion**

Our results show that the mutations of *porD* and *oorD* in *H. pylori* may be associated with resistance to furazolidone. The physiology and metabolism of *H. pylori* are not well understood (15). *H. pylori* has enzymes of the Entner-Doudoroff and pentose-phosphate pathways (16). However, the major routes for the generation of acetyl coenzyme A (acetyl-CoA) and succinyl-CoA are via pyruvate:flavodoxin oxidoreduc-



**Figure 3.** Analysis of *porD* mutations from furazolidone-resistant strains of *Helicobacter pylori* showed that 353 glutamate was replaced by aspartate, 356 alanine by threonine, and 357 threonine by valine.



**Figure 4.** Analysis of *oorD* mutations from furazolidone-resistant strains of *Helicobacter pylori* showed that 041 threonine was replaced by alanine, 122 isoleucine by valine, and 349 asparagine by lysine.

tase (POR) and 2-oxoglutarate:acceptor oxidoreductase (OOR), respectively. These two electron acceptors may be ferredoxin (Fd) or flavodoxin (FldA). Both *porD* and *oorD* encode integral ferredoxin-like subunits (17). Furazolidone is a nitrofurans antibiotic. For nitrofurans, the spectrum of bioactivity and the mechanism of activation mainly depend on the redox potential of the 5-nitro groups. Furazolidone shows relatively high redox potentials (-250 to -270 mV) and is reductively activated by a wide range of nitroreductases (18). Putative *H. pylori* nitroreductases include FdxB, FrxA, RdxA, OorD, and PorD. Kwon DH et al (19) found that the inactivation of *fdxB*, *frxA*, and *rdxA* genes did not cause a lethal effect on *H. pylori*, but that both *porD* and *oorD* seem to be necessary for survival of *H. pylori*. They also discovered that RdxA, FrxA, and FdxB could not deoxidize furazolidone. The *rdxA*, *frxA*, and *fdxB* genes, which were knocked out, did not result in the resistance of *H. pylori* to furazolidone (10). Therefore, Kwon et al (19) suggested that *por* and *oor* genes might be crucial for the resistance of *H. pylori* to furazolidone.

The extensive use of furazolidone results in increasing resistance rate of *H. pylori*. In addition, the resistance rate seems to vary from one geographic region to another (20). For example, Shan et al (21) reported *H. pylori* resistance rate to furazolidone of up to 40% in Shanghai, in Southeastern China. On the other hand, Liu

et al (22) reported that all *H. pylori* isolates were susceptible to furazolidone in Shijiazhuang city, in Northern China. Our results showed that *H. pylori* resistance rate was 8.7% in Zhenjiang, which is located in the south of Yangtze River, supporting the previous observation of geographic variations in *H. pylori* resistance rate. The major reasons for this difference may be due to different lifestyle in different regions. For example, people living in southeast China eat lot of fish. Since furazolidone is often used as an antiseptic in fishponds, it can lead to the accumulation of the drug in fish and increased exposure of the population living in that part of China, leading to the observed high *H. pylori* resistance rate to furazolidone.

Sisson et al (23) discovered that partially inhibitory (near MIC) concentrations of furazolidone were somewhat mutagenic for *H. pylori*, suggesting that the *porD* and *oorD* mutations of resistant and intermediately susceptible strains might have relations with furazolidone bioactivity. In the present study, we found that both *porD* and *oorD* of resistant strains had three mutations each. These mutations in both genes may contribute to the resistance of *H. pylori* to furazolidone, which is a finding consistent with observations by Kwan et al (19).

In conclusion, we found a relatively high rate of *H. pylori* resistance to antibiotics in Zhenjiang, China. This finding is in accordance with

previous observations that *H. pylori* resistance significantly varies with geographic region in China. Furthermore, our results showed the association between mutations in *porD* and *oorD* genes of *H. pylori* and its resistance to furazolidone. These findings may provide useful information to a clinician administering antibiotics for the treatment of *H. pylori* infection. However, further research is needed to understand the exact resistance mechanism of *H. pylori* to furazolidone.

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