

ANTIMICROBIAL RESISTANCE

## Primary and combined resistance to four antimicrobial agents in *Helicobacter pylori* in Sofia, Bulgaria

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The aim of this study was to evaluate the primary and combined resistance of *Helicobacter pylori* against four antimicrobial agents by a screening agar method (SAM) and a modified disk diffusion method (MDDM) alone and in combination. Pre-treatment *H. pylori* isolates from 192 consecutive *H. pylori*-positive patients at three hospitals in Sofia were investigated. MDDM was performed with disks containing metronidazole (5 µg), clarithromycin (15 µg) or erythromycin (15 µg), ciprofloxacin (5 µg) and tetracycline (30 µg). Resistance was determined by an inhibitory zone of <16 mm for metronidazole and ≤30 mm for other agents tested. The cut-off concentrations used to define resistance by SAM were: metronidazole >8 mg/L, clarithromycin >2 mg/L, tetracycline >4 mg/L and ciprofloxacin >1 mg/L. Primary resistance rates in *H. pylori* were: metronidazole 28.6%, clarithromycin 9.7%, metronidazole + clarithromycin 2.8%, ciprofloxacin 3.9%, metronidazole + ciprofloxacin 2.3%, tetracycline 1.9% and metronidazole + tetracycline 1.2%. Among metronidazole-resistant isolates, combined resistance to clarithromycin, ciprofloxacin and tetracycline was present in 11.4% (5 of 44 strains), 8.3% (3 of 36) and 4.9% (2 of 41), respectively. Two strains exhibited triple resistance to macrolides, metronidazole and either ciprofloxacin or tetracycline. Three tetracycline-resistant strains were detected in 1999; however, resistance rates to other agents were relatively stable during the 6 years. Primary *H. pylori* resistance to metronidazole is moderate and resistance to clarithromycin and to ciprofloxacin is considerable in comparison with results in most other countries. The alarming appearance of strains harbouring combined resistance or multiresistance provides the motivation for continued surveillance of *H. pylori* at global, national and regional levels.

### Introduction

*Helicobacter pylori* infection causes chronic gastritis, which can trigger peptic ulcer disease and gastric malignancies in man [1]. Successful eradication of the infection results in ulcer healing and prevents ulcer recurrences and complications [2]. Primary resistance in *H. pylori* has been reported to nitroimidazoles (in 6–95% of isolates), macrolides (0–17%), ciprofloxacin (0–1%) and tetracycline (0–6%) in different countries

[2–8]. Both primary and secondary resistances of the bacterium are major reasons for therapeutic failure and require constant monitoring [2, 9]. For this purpose, several methods for routine susceptibility testing of *H. pylori* have been evaluated, including the E test, modified disk diffusion method (MDDM) and screening agar method (SAM), and the standardisation of procedures is gradually progressing [9, 10]. However, there is a lack of interpretive category standards for both minimal inhibitory concentrations (MICs) and inhibitory zone diameters for resistance to many antibiotics in *H. pylori*. The aim of the present study was to assess the primary resistance rates of clinical isolates of *H. pylori* to four antimicrobial agents and the combined resistance of the bacterium to more than one drug.

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## Materials and methods

### Patients and biopsy specimens

*H. pylori* strains were obtained from gastric biopsy specimens of 192 consecutive *H. pylori*-positive patients (113 men, 79 women; average age 30.2 years, range 3–76 years) with peptic ulcers (77 patients) and chronic gastritis (115 cases). The specimens were collected from patients undergoing routine gastrointestinal tract endoscopy at three hospitals in Sofia from June 1993 to June 1999. None of the patients had been treated with metronidazole, macrolides, tetracyclines or fluoroquinolones for the eradication of *H. pylori* infection. The strains were isolated and identified by standard procedures as described previously [11], and susceptibility testing was performed by MDDM or SAM, and by both methods in 70 cases.

### Modified disk diffusion method (MDDM)

*H. pylori* strains were inoculated on to Brucella Blood Agar plates (Oxoid, Basingstoke, or Merck, Darmstadt, Germany) with Isovitalex (Becton Dickinson, BBL Microbiology Systems, Cockeysville, MD, USA) 1%. Colonies were suspended in sterile saline and adjusted to a density equal to McFarland turbidity standard 3–4. The suspensions were spread on to the plates with sterile cotton swabs and then disks containing metronidazole (5 µg), clarithromycin (15 µg), ciprofloxacin (5 µg) or tetracycline (30 µg) were added. The clarithromycin susceptibility of 34 isolates was tested by erythromycin disks (15 µg) because of the cross-resistance with all macrolides [4]. Fauchere [12] recently reported a 100% concordance between susceptibility to erythromycin and clarithromycin in *H. pylori*. The plates were incubated micro-aerobically (Campylobacter Gas Generating Kits BR 060A, Oxoid, or Campy Pak Plus, Becton Dickinson) at 37°C for 3 days. Resistance was determined by a zone of growth inhibition <16 mm corresponding to an MIC >8 mg/L for metronidazole [13], and zone diameters ≤30 mm for clarithromycin or erythromycin, ciprofloxacin and tetracycline [8]. Greater zones of complete growth inhibition indicated the presence of susceptible strains. The MDDM susceptibility tests were performed in

duplicate and median diameters of growth inhibition were taken into account.

### Screening agar method (SAM)

Two drops (c. 60 µl) of *H. pylori* suspensions prepared as above were inoculated on one-quarter of the surface of Brucella Blood Agar plates containing one of the following drugs: metronidazole 8 mg/L, clarithromycin 2 mg/L, ciprofloxacin 1 mg/L or tetracycline 4 mg/L. Metronidazole and tetracycline were obtained from Sigma, clarithromycin from Abbott Laboratories (Chicago, IL, USA) and ciprofloxacin from Bayer Pharma (Sens, France). The plates were incubated micro-aerobically at 37°C for 3 days. If *H. pylori* growth appeared on the plate, the isolate was deemed to be resistant to the corresponding drug. Non-selective plates were used as a control of strain viability. If resistance was detected by either SAM or MDDM or by both methods, the strain was considered to be resistant to the corresponding antimicrobial agent.

### Statistical analysis

Differences between patients with susceptible and resistant strains were assessed by the  $\chi^2$  test with or without Yates's correction.

## Results

Primary resistance rates in *H. pylori* were as follows: metronidazole 28.6%, clarithromycin 9.7%, ciprofloxacin 3.9% and tetracycline 1.9% (Table 1). There were no differences ( $p > 0.1$ ) in the antimicrobial resistance rates and diseases (peptic ulcer *versus* chronic gastritis) or different age groups (patients <17 years old *versus* patients ≥18 years old). Although women more frequently harboured strains resistant to metronidazole (40.5% *versus* 20.4% in men), clarithromycin (11.4% *versus* 8.6%) and ciprofloxacin (6.1% *versus* 2.5%), the differences were significant ( $p < 0.01$ ) only in regard to metronidazole.

Combined resistance to metronidazole and either macrolides or fluoroquinolones was found in five of

**Table 1.** Primary and combined resistance of *H. pylori* to antimicrobial agents

Agents	Number of strains	Number (%) of resistant strains
Metronidazole	192	55 (28.6)
Clarithromycin	175	17 (9.7)
Ciprofloxacin	129	5 (3.9)
Tetracycline	160	3 (1.9)
Metronidazole + clarithromycin	175	5 (2.8)
Metronidazole + ciprofloxacin	129	3 (2.3)
Clarithromycin + ciprofloxacin	121	2 (1.6)
Metronidazole + clarithromycin + ciprofloxacin	121	1 (0.8)
Metronidazole + tetracycline	160	2 (1.2)
Metronidazole + clarithromycin + tetracycline	160	1 (0.6)

175 strains (2.8%) and in three of 129 strains (2.3%), respectively, and two of 160 isolates (1.2%) were not susceptible to both metronidazole and tetracycline. Among metronidazole-resistant isolates, combined resistance to clarithromycin, ciprofloxacin and tetracycline was observed in 11.4%, 8.3% and 4.9% respectively (Table 2). Clarithromycin-resistant isolates were not susceptible to metronidazole, ciprofloxacin and tetracycline in 26.7%, 20.0% and 7.7%, respectively. One strain exhibited multiple resistance to clarithromycin, metronidazole and ciprofloxacin. This strain was isolated from the gastric mucosa of a 56-year-old woman with duodenal ulcer. One isolate that was resistant to tetracycline, clarithromycin and metronidazole came from a 25-year-old man with chronic gastritis.

The metronidazole resistance rate was stable, being 28.1% in 1993–1996 and 27.3% in 1998–1999 ( $p > 0.20$ ). Incidences of resistance to macrolides (8.2% in 1993–1996 and 11.5% in 1997–1999) and to ciprofloxacin (2.1% in 1993–1996 and 9.1% in 1997–1999) showed a slight tendency to increase; however, the differences were not statistically significant ( $p > 0.20$ ). Tetracycline resistance was detected only in 1999.

## Discussion

Resistance of *H. pylori* to antimicrobial agents is the main cause of treatment failure. Even a triple regimen (including amoxicillin, clarithromycin and omeprazole) has usually led to *H. pylori* eradication for 50% of patients infected with clarithromycin-resistant strains versus >90% for those harbouring susceptible isolates [3, 9]. The clinical relevance of metronidazole resistance detected *in vitro* is more controversial. In one study, a triple scheme using amoxicillin, metronidazole and lansoprazole has been associated with a 96% cure rate for isolates with metronidazole MICs <2 mg/L versus 45% for those with MICs  $\geq$ 16 mg/L; however, other authors have reported better eradication rates with similar regimens [3, 9].

*H. pylori* is a fastidious and slow growing microaerophilic bacterium, and its susceptibility testing has specific requirements. For this purpose, the breakpoint method (8 mg/L) has been recommended for metronidazole and different techniques are applicable for

macrolides [9]. Although it is generally accepted that clarithromycin 2 mg/L and metronidazole 8 mg/L correspond to the breakpoints for *H. pylori* resistance [13–16], MIC interpretive standards for other antimicrobial agents are still lacking. Moreover, several authors have proposed an intermediate category for *H. pylori* strains inhibited by metronidazole 8 mg/L or exhibiting zone diameters of 16–<21 mm [13]. According to another study, the metronidazole intermediate category has been proposed for isolates with inhibitory zone diameters of 20–26 mm (MIC 4–8 mg/L) [14]. These studies emphasise that metronidazole susceptibility testing of this organism is a great challenge and the results must be interpreted with caution.

The present study used tetracycline 4 mg/L for SAM susceptibility testing because this concentration is recommended by the NCCLS for determining susceptible strains in anaerobic microbiology [17]. The cut-off level for ciprofloxacin-susceptible strains (1 mg/L) was chosen because sensitive *H. pylori* isolates have exhibited MICs  $\leq$ 0.5 mg/L and resistant strains have shown ciprofloxacin MICs >2 mg/L, according to Megraud [9].

The disk diffusion method is usually considered inappropriate for slow-growing organisms, because the gradient of drug concentration in the agar after prolonged incubation is not reproducible [18]. However, this technique is applicable for testing the susceptibility of *H. pylori* to antibiotics when there is a clearly bimodal population (e.g., clarithromycin) [3]. Different authors [13–15] have also reported MDDM to be a reliable method for metronidazole susceptibility testing. In the present study strains were considered to be resistant to metronidazole by MDDM according to the interpretive criteria of Chaves *et al.* [13]. The narrow range of MICs and homogeneous susceptibility of *H. pylori* to clarithromycin, ciprofloxacin and tetracycline have been reported [10]; therefore, large inhibitory areas (>30 mm) seem to be most suitable for detecting isolates which are susceptible to all drugs except metronidazole.

A considerable difference has been observed between the rates of primary metronidazole resistance of *H. pylori* in developed (6–49%) and developing (77–95%) countries because of the extensive use of nitroimidazoles for treating parasitic diseases in the

**Table 2.** Combined resistance in primary resistant *H. pylori* isolates

Strains resistant to	Combined resistance (%) to			
	Clarithromycin	Ciprofloxacin	Metronidazole	Tetracycline
Metronidazole	5 of 44 (11.4)	3 of 36 (8.3)	...	2 of 41 (4.9)
Clarithromycin	...	2 of 10 (20.0)	4 of 15 (26.7)	1 of 13 (7.7)
Ciprofloxacin	2 of 4 (50.0)	...	3 of 5 (60.0)	0 of 5 (0)
Tetracycline	1 of 3 (33.3)	NT	2 of 3 (66.7)	...

NT, not tested.

latter countries [2–5]. In the present study, the prevalence was similar to the median rates in Europe, and encompassed *c.* 29% of the pre-treatment isolates. On the contrary, the rate of clarithromycin resistance was higher than that observed in Germany and the Netherlands (1–2%) and was similar to that reported in France (10%) [2, 3, 5, 6]. Mégraud [9] has emphasised that in the case of resistance rates <15% to clarithromycin and <30% to metronidazole, these agents could be used in therapeutic regimens without in-vitro susceptibility testing of *H. pylori*. The present results demonstrated that primary *H. pylori* resistance to metronidazole is close to the crucial 30% and that to macrolides is also worrying.

Although ciprofloxacin is not a drug of choice in the therapy of *H. pylori* infection, its combination with amoxicillin could be considered as an alternative in case of combined resistance of the bacterium to the first-line antimicrobial agents [9]. In comparison with the low prevalence ( $\leq 1\%$ ) of ciprofloxacin resistance in *H. pylori* in most studies [3, 7] the present study found a striking (3.9%) resistance rate. Carbone *et al.* [19] have reported high prevalence of fluoroquinolone resistance in *H. pylori* as well, three of the 32 strains tested being resistant to ciprofloxacin >5 mg/L. In the present work, all isolates were uniformly susceptible to tetracycline except three resistant strains detected in 1999. Resistance rates to the other agents tested were relatively stable over the 6 years of the study. Primary antibiotic resistance rates exhibited no association with age and clinical diagnosis. A significantly higher incidence of metronidazole resistance was observed in women than in men, as reported previously [4].

Approximately 1–3% of the pre-treatment isolates exhibited double resistance to different drugs. Combined resistance to metronidazole and clarithromycin was often detected among macrolide-resistant isolates (*c.* 25%) and affected around 10% of the metronidazole-resistant strains. Simultaneous primary resistance to these agents was reported in four of six clarithromycin-resistant isolates by van Zwet *et al.* [5], and such strains have been associated with therapy failure [2]. The problem concerning combined and multiple resistance in *H. pylori* is of utmost importance, as the efficacy of the current therapeutic regimens may be compromised. Moreover, the present study detected two pre-treatment isolates showing triple resistance to metronidazole, clarithromycin and either ciprofloxacin or tetracycline. Nakae *et al.* [20] found triple resistance in 14.3% and double resistance in 28.6% of *H. pylori* strains resistant to macrolides, fluoroquinolones or nitroimidazoles.

In conclusion, the present results emphasise the need to perform constant monitoring of *H. pylori* resistance patterns to antimicrobial agents at global, national and regional levels in order to counteract the tendencies in the evolution of resistance.

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