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TITLE

Antimicrobial susceptibility of *Helicobacter pylori* against six currently used antibiotics in Spain

Running title: Searching for appropriate *H. pylori* eradication antibiotics

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SYNOPSIS

Background: Antibiotic resistance is directly related to the loss of efficacy of currently accepted *Helicobacter pylori* therapies. The knowledge of the antibiotic susceptibility in a local area can contribute to design specific "*à la carte*" treatments. The aim of this study was to analyze the susceptibility pattern of *H. pylori* isolates regarding to six conventional antibiotics currently used in a northern region of Spain.

Materials and methods: Seventy-one isolates were obtained from gastric biopsies of 76 consecutive adult patients suffering from peptic ulcer disease, dyspepsia or familiar gastric cancer and known to be infected with *H. pylori* by conventional methods. Susceptibility testing was performed for amoxicillin, ciprofloxacin, levofloxacin, clarithromycin, metronidazole, and tetracycline by using the Etest method.

Results: The prevalence rates of resistance were as follows: amoxicillin, 1.4% (95% Confidence Interval [CI], 0.0 to 7.6); clarithromycin, 14.7% (CI, 7.3 to 25.4); ciprofloxacin, 14.3% (CI, 7.1 to 24.7); levofloxacin, 14.5% (CI, 7.2 to 25.0); metronidazole, 45.1% (CI, 33.2 to 57.3); and tetracycline, 0% (CI, 0.0 to 5.1).

Conclusions: Our study confirms an increasing rate of resistance to levofloxacin which equals that of clarithromycin in our health care area. This fact may reflect a wide and indiscriminate use of the former antibiotic and could account for a loss of clinical effectiveness of levofloxacin-containing regimens. Moreover, resistance rates against clarithromycin remain stable which could allow us to maintain its use in our area.

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INTRODUCTION

Helicobacter pylori infects the gastric mucosa and represents the main cause of gastritis, peptic ulcer disease, and gastric cancer. It has been demonstrated that eradication of *H. pylori* improves the clinical outcome of patients with duodenal ulcer, prevents its recurrence, and decrease the risk of gastric cancer in infected patients.[1,2] Nowadays, the European and American guidelines on the treatment of *H. Pylori* infection recommend as first-line therapy a combination of a proton pump inhibitor (PPI) with two antibiotics, being omeprazole and clarithromycin plus amoxicillin or metronidazole the preferred regimen.[3,4] Although the initial eradication success rate for the standard triple therapy was in excess of 90% ten years ago, therapy failures up to 30-40% of cases using this regimen have been more recently reported.[5-8] Even though patient's lack of compliance, inadequate length of therapy, or a high bacterial burden are conditions that may contribute to such loss of efficacy, antimicrobial resistance is regarded as the leading factor responsible for eradication failure. This issue is of particular relevance in regard to clarithromycin which can induce a virtually 70% loss of effectiveness when takes part of an PPI-amoxicillin based triple therapy, depending on *in vitro* macrolide susceptibility.[9] In fact, the most recent Maastricht guidelines on *H. pylori* infection management recommend substituting metronidazole for clarithromycin when resistance to this antibiotic exceeds 15-20%.[3] Finally, there is a wide geographic variation regarding the prevalence of antibiotic resistance. This fact has been recently highlighted in the updated European Surveillance of *H. pylori* Resistance to Antibiotics where differences in clarithromycin resistance rates of more than 10% were detected between different regions of Europe, precluding its use in some of them.[10] All of that makes general guidelines related to the use of different antibiotic regimens

against *H. pylori* useless if they do not include the available data about antibiotic susceptibility in local areas. Taking into account the aforementioned considerations it seems desirable to have regularly updated, reliable information on the prevalence of antibiotic resistance to *H. pylori* for the various countries, regions or health care areas. Such information can aid to establish on an individual basis the potentially most effective eradicating regimen for *H. pylori* infection.[11-13]

The aim of this study was to assess the susceptibility of *H. pylori* strains isolated from gastric biopsies of patients with gastroduodenal peptic ulcer disease, treatment unresponsive dyspepsia, or family history of gastric cancer to six antibiotics commonly used in therapeutic procedures.

MATERIAL AND METHODS

Patients and sample collection

From February to December, 2010, seventy-six consecutive adult patients who had not been previously eradicated against *H. pylori* were evaluated at the Gastroenterology Department of the Hospital of Laredo, a community hospital placed on the North of Spain. These patients referred different upper abdominal complaints, a familiar history of gastric cancer and/or a personal history of gastroduodenal peptic ulcer disease. All of them underwent a diagnostic esophagogastroduodenoscopy including collection of biopsies of the gastric mucosa (from both the body and the antrum) for rapid urease test, histologic study, and culture.

Culture preparation and susceptibility testing

The biopsy specimens of those patients with a positive urease test were homogenized and sowed in selective (Agar Pylori-BioMérieux, Sweden) and no selective (Columbia III Agar with 5% Sheep Blood, Becton-Dickinson, Germany) culture media and incubated at 37ºC under microaerophilic conditions for 10-14 days. Once cultures were obtained, the isolates were identified according to colonial morphology, Gram-staining, urease, catalase and oxidase tests. Afterwards, the strains were re-sowed and subcultured in non-selective media (Columbia III Agar with 5% Sheep Blood) for 48-72 hours in order to perform the susceptibility studies. The minimum inhibitory concentration (MIC) was determined using the Etest method as recommended by the *British Society for Antimicrobial Chemotherapy* (BSAC).[14] Muller Hinton agar supplemented with 5% sheep blood (Becton-Dickinson, Germany) was used as culture media. The *H. pylori* culture suspension of 3.0

McFarland turbidity was used to inoculate the plates by confluent swabbing and Etest strips (BioMérieux, Sweden) were applied onto culture plates. The plates were incubated at 35ºC for 3-5 days under microaerophilic conditions. The tested drugs were amoxicillin, clarithromycin, ciprofloxacin, levofloxacin, metronidazole, and tetracycline. The breakpoints used to classify strains as susceptible or resistant according to the MIC value were as follows: ≤1 mg/L, susceptible (S) and ≥2 mg/L, resistant (R) for amoxicillin and clarithromycin; ≤1 mg/L, S and ≥1 mg/L, R for ciprofloxacin and levofloxacin; ≤4 mg/L, S and ≥8 mg/L, R for metronidazole; and ≤2 mg/L, S and ≥4 mg/L, R for tetracycline. The breakpoints for amoxicillin, clarithromycin, metronidazole, and tetracycline were interpreted according to the BSAC recommendations. Quinolones are not standardised by the BSAC so we used those recommended by the *Societé Française de Microbiologie* that are in accordance with those suggested by other authors.[15,16]

Statistics

A descriptive analysis was performed using the v.15.0 SPSS package.

Ethics

This study was performed following the current standards of Good Clinical Practice and Good Laboratory Practice and the protocol was approved by the Cantabric Ethical Investigation Committee. An informed consent was obtained from all the patients.

RESULTS

Seventy-one *H. pylori* strains were isolated from 76 consecutive adult patients (31 male and 45 female) who had been included in the study on the basis of a positive rapid urease test. Susceptibility figures corresponding to the six tested antimicrobials were determined according to the aforementioned Etest method and are summarised in Table 1 (in 4 of 71 isolates antimicrobial susceptibility could not be tested for all antibiotics).

Forty-four out of 71 isolates (62%) showed resistance to at least one antibiotic, while in 27 patients all isolates were susceptible to the tested antibiotics. Resistance to only one antibiotic was present in the isolates obtained from thirty-one patients. On the other hand, 13 isolates showed resistance to 2 or more antibiotics, mainly involving quinolones (5 patients). Isolates coming from five patients showed multi-resistance, defined as having resistance to 3 or more antibiotics (in four patients, *H. pylori* strains exhibited resistance to ciprofloxacin, levofloxacin and metronidazole, and in another patient the isolate was also resistant to clarithromycin). Finally, only 1 patient had an isolate that was simultaneously resistant to levofloxacin and clarithromycin.

DISCUSSION

The knowledge of the available data about *in vitro* antimicrobial susceptibility of *H. pylori* has been requested by several experts in order to increase the therapeutic success rate for *H. pylori* eradication.[11,13] The rationale for this claim relies on two facts: first, the well-known variability on the prevalence of antibiotic resistance among different countries or even regions or social groups (mainly related to clarithromycin); and second, the special consequences that a wrong antibiotic selection can bear, as it has been stated before (for example, a nearly 70% loss of efficacy depending on susceptibility or resistance to clarithromycin).

In the present study, we have found a wide spectrum of resistance rates of *H. pylori*, from nearly negligible figures against tetracycline (0%) and amoxicillin (1.4%) to high resistance rates against metronidazole (45.1%). Intermediate and virtually identical figures were found for clarithromycin (14.7%), ciprofloxacin (14.3%), and levofloxacin (14.5%). These results merit some considerations. Firstly, the prevalence rates of *H. pylori* resistance against clarithromycin and amoxicillin are in accordance to those reported in 2001 in Madrid (Spain) within the European Multicentre Survey of *in vitro* Antimicrobial Resistance in *H. pylori* (15% and 0%, respectively).[17] It is important to remark the scarce variation on resistance patterns found throughout the last decade, particularly in regard to clarithromycin, which could stand for a maintained relatively high efficacy of the standard triple therapy against *H. pylori* in our region. These results are in accordance with those reported in a recent European survey, where clarithromycin resistance rates were 17% as a whole.[10] While countries from de Centre, West, and South of Europe have experienced a great increase in resistance rates against clarithromycin (>20%), which jeopardizes its use as part of conventional triple therapy empirical regimens,

northern countries and other exceptions like Germany and Spain maintain low-intermediate resistance rates. Our group is in the process of conducting a randomised clinical trial on first-line *H. pylori* eradication therapy comparing two triple therapy based regimens (namely omeprazole, amoxicillin, and either clarithromycin or levofloxacin) which will help us to clarify the true clinical effectiveness of clarithromycin and levofloxacin in our area. A high rate of eradication with standard first-line therapies has been achieved in other regions on the basis of a high adherence to the treatment so a combination of moderate slowly-growing rate of resistance to antibiotics and high levels of compliance could account for a sustained high eradication rates.[8,18] Secondly, the resistance rates to metronidazole are slightly higher than previously reported in Spain (37.2%) and in Europe (33.1%) in 2001, approaching to those encountered in Italy (49%), Austria (44.9%), and Greece (44.1%).[17] Certainly, metronidazole resistances have remained stable in the last decade in Europe as a whole (34.3%; CI, 16.7 to 50.3)[10] although individual figures of each country are awaited and differences between them as those found for clarithromycin can be expected to confirm our results. Even though *in vitro* resistance to metronidazole may not accurately reflect *in vivo* resistance, regimens including metronidazole could not be a preferable choice in populations with >40% metronidazole resistance.[12,19] Thus, our data could dissuade gastroenterologists of our region to use this antibiotic as taking part of an alternative first-line therapeutic regimen against *H. pylori*, particularly in cases of penicillin allergy.

Thirdly and of great interest in this study is the notable *H. pylori* resistance rate against levofloxacin, a quinolone increasingly used as a clarithromycin-substitute for either first-line or rescue therapy in different regimens.[20] In spite of the high eradication rates (~90%) achieved with the combination of PPI, amoxicillin, and

levofloxacin, there are concerns about an increasing rate of quinolone resistance: 15% in Japan, 16.8% in Belgium, 23.1% in Italy, from 2.8% to 11.8% between 1998 and 2003 in Taiwan, from 3% in 1999 to 15% in 2004 in France, and from 11.2% in 2003 to 22.1% in 2005 in Germany. [8,16,20,21-24] These changes on *H. pylori* susceptibility to quinolones could account for a fall in the success rate of a triple therapy including levofloxacin. In fact, some investigators have linked the slight reduction in overall eradication rates of a levofloxacin-based re-treatment (from 76-85.7% to 72.7%) to the high prevalence of *in vitro* primary resistance (30.3%) which doubled that found in previous trials.[20] Although a relatively low rate of *H. pylori* resistance to quinolones (6%) had been previously reported in Spain,[25] the present study reveals and confirms an increasing rate of levofloxacin resistance in our country which is similar to that has been reported in other Mediterranean areas. In this way, the aforementioned European study on antibiotic resistance of *H. pylori*[10] underscores a progressive trend to a higher resistance rates to levofloxacin (similar to those encountered in our study) which can discourage the future use of eradication regimens including this quinolone. Finally, resistances against amoxicillin and tetracycline remain negligible.

To sum up, the present work shows stable *in vitro* resistance rates of *H. pylori* to clarithromycin which could support its use as a part of *H. pylori* eradication regimens in our area. In addition, it also confirms a quick increase of the *in vitro* resistance rate to levofloxacin in our region which may discourage its use in eradication regimens, at least as first-line treatment. Taking into account the important variability of prevalence rates of *H. pylori* resistance to different antibiotics (in time and space) and the consequences that this fact can bring on therapy success we encourage regional Gastroenterology and Microbiology Societies

to periodically update their data on *in vitro* resistances and then make suitable recommendations about the best desirable therapy.

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TRANSPARENCY DECLARATION

None to declare.

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Table 1. Antimicrobial susceptibility of *H. pylori* isolates in the North of Spain

Antibiotic	Isolates tested (n)	Isolates with resistance (n)	Resistance, % (95 CI)	MIC	
				S	R
Amoxicillin	71	1	1.4 (0.0-7.6)	≤1	≥2
Ciprofloxacin	70	10	14.3 (7.1-24.7)	≤1	≥1
Levofloxacin	69	10	14.5 (7.2-25)	≤1	≥1
Clarithromycin	68	10	14.7 (7.3-25.4)	≤1	≥2
Metronidazole	71	32	45.1 (33.2-57.3)	≤4	≥8
Tetracycline	71	0	0.0 (0.0-5.1)	≤2	≥4

MIC = Minimal Inhibitory Concentration (mg/L). This parameter was established for each antibiotic as breakpoints of susceptibility (S) or resistance (R); **n** = number; **95 CI** = 95% Confidence Interval.