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Helicobacter pylori antimicrobial resistance rates in the central region of Portugal

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Abstract

Helicobacter pylori resistance to antimicrobial agents is steadily increasing. It is extremely important to be aware of the local prevalence of antibiotic resistance so as to adjust treatment strategies. During this single-centre, prospective study, we aimed to determine primary and secondary resistance rates of *H. pylori* to antibiotics as well as host and bacterial factors associated with this problem. Overall, 180 patients (131 female; mean age 43.4 ± 13.5 years; primary resistance 103; secondary resistance 77) with positive ^{13}C-urea breath test were submitted to upper endoscopy with gastric biopsies. *Helicobacter pylori* was cultured and antimicrobial susceptibility was determined by Etest and molecular methods. Clinical and microbiological characteristics associated with resistance were evaluated by logistic regression analysis. Among the 180 isolates 50% were resistant to clarithromycin (primary 21.4%; secondary 88.3%), 34.4% to metronidazole (primary 29.1%; secondary 41.6%), 33.9% to levofloxacin (primary 26.2%; secondary 44.2%), 0.6% to tetracycline and 0.6% to amoxicillin. Being female was an independent predictor of resistance to clarithromycin and metronidazole. Previous, failed, eradication treatments were also associated with a decrease in susceptibility to clarithromycin. History of frequent infections, first-degree relatives with gastric carcinoma and low education levels determined increased resistance to levofloxacin. Mutations in the *23S rRNA* and *gyrA* genes were frequently found in isolates with resistance to clarithromycin and levofloxacin, respectively. This study revealed that resistance rates to clarithromycin, metronidazole and levofloxacin are very high and may compromise *H. pylori* eradication with first-line and second-line empiric triple treatments in Portugal.

Keywords: Antibiotics, *Helicobacter pylori*, multidrug resistant, mutations *23S rRNA*, mutations *gyrA*, primary resistance, secondary resistance
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Introduction

Helicobacter pylori is responsible for multiple gastric pathologies [1]. The universally accepted empiric triple treatment for *H. pylori* comprises proton-pump inhibitors (PPI), clarithromycin and amoxicillin or metronidazole [1]. However, the efficacy of the 'legacy' triple therapy has decreased in the last decades and is now inferior to 80% in many countries [1,2]. There are many reasons for therapeutic failure including poor compliance, inadequate dose/duration of therapy, rapid metabolism of PPI, ineffective penetration of antibiotics into the gastric mucosa, antibiotic inactivation by low gastric pH and, most importantly, *H. pylori* resistance to antimicrobial agents [3,4]. Excessive and indiscriminate use of antibiotics is leading to decreased susceptibility of *H. pylori* to such drugs [5].

Resistance of *H. pylori* to antibiotics is common in clinical practice but we must distinguish primary from secondary resistance. Exposure of *H. pylori* to antibiotics during eradication attempts can select bacterial resistance. This occurs

mainly with clarithromycin and levofloxacin but also with nitroimidazoles. Reports about primary resistance are common but data after therapeutic failure is scarcer because patients previously exposed to anti-*H. pylori* treatment are frequently excluded from susceptibility studies [6,7].

Prevalence of *H. pylori* infection in Portugal is 84.2%, 66.2% and 31.6% for adults, teenagers and children, respectively [8–10]. In 2012 the estimated age-standardized incidence and mortality rates for stomach cancer in Portugal were still some of the highest in Europe [11]. For this reason, *H. pylori* will continue to represent a major healthcare problem in this south European country. The European multicentre studies of *in vitro* antimicrobial resistance revealed that in Portugal primary *H. pylori* resistance rates to clarithromycin, levofloxacin and metronidazole were 31.5%, 26.3% and 33.3%, respectively [5,7]. However, there is little information concerning secondary resistance rates and to our knowledge there are no data about this problem in the central region of the country.

The focus of the present study was to evaluate primary and secondary resistance rates of *H. pylori* to antimicrobials in this region of Portugal. As a secondary objective, we intended to establish potential host and bacterial factors associated with resistance to each one of the tested antibiotics.

Patients and Methods

Patients

In this single-centre study patients with dyspepsia, iron-deficient anaemia, need for chronic therapy with PPI and/or first-degree relatives with gastric carcinoma were prospectively considered for inclusion, from September 2009 to October 2013. All of them had a positive ^{13}C-urea breath test. Exclusion criteria were: age <18 years; pregnancy; lactating and/or fertile women who were not using safe contraceptive methods; history of allergy/hypersensitivity to any antibiotic or PPI; previous gastric malignancy and/or gastric surgery; current use of anticoagulants; marked thrombocytopenia; systemic severe disease; use of antibiotics in the last 4 weeks or PPI in the last 2 weeks.

Patients were divided into two groups: Group I—no previous *H. pylori* eradication treatment (primary resistance); Group II—previous, failed *H. pylori* therapy (secondary resistance).

Study design

All patients were submitted to upper endoscopy with biopsies in the antrum and corpus that were immediately placed in independent containers of adequate transport media—Portagerm pylori (bioMérieux Portugal, Linda-A-Velha, Portugal)—at 4°C, and sent to the microbiology laboratory. Urease test and Gram staining of a smear prepared from the biopsy specimen confirmed the presence of *H. pylori*. After manual grinding with disposable material the samples were distributed directly in agar pylori (bioMerieux Portugal, Linda-A-Velha, Portugal). Cultures were incubated for a minimum of 72 h and a maximum of 10 days at 37°C under microaerobic conditions, produced with H_2-CO_2-generating packs (GENbag, bioMérieux Portugal, Linda-A-Velha, Portugal). The *H. pylori* isolates were identified by colony morphology, characteristic spiral morphology on Gram staining, and positive catalase, urease and oxidase tests. Antrum and corpus samples were processed separately.

The MIC for amoxicillin, clarithromycin, metronidazole, levofloxacin and tetracycline were determined by Etest (bioMérieux Portugal) and expressed in mg/L. To minimize variations in the results, *H. pylori* ATCC 43504 was used for quality control of the susceptibility assay and a different microbiologist, blinded to previous results, repeated all tests. Strains were considered resistant to amoxicillin, clarithromycin, metronidazole, levofloxacin and tetracycline at MIC >0.5, >1, >8, >1, and >1 mg/L, respectively. MIC values were established according to the data available in 2009, including the CLSI breakpoints [3,12–15]. A strain was considered multidrug-resistant if it had resistance to two or more antibiotics.

DNA extraction from pure culture of *H. pylori* was performed with a special extraction kit (QIAamp® DNA Mini Kits, QIAGEN, Izasa Portugal, Carnaxide, Portugal) according to the manufacturer's instructions. Point mutations in *23S rRNA* (A2143G/A2142G, A2142C), the quinolone resistance-determining region (QRDR) of the *gyrA* and the *16S rRNA* genes were detected by real-time PCR using a LightCycler device, as previously described [13,16,17].

The *cagA*, *vacA*, *iceA* and *babA2* genotypes were determined with real-time PCR by using specific primers selected from previously published works [18–20].

Statistics

Categorical variables were presented with their relative and absolute values and quantitative ones were expressed as mean ± standard deviation or median + range. For statistical analysis, Student's *t*-test, Mann–Whitney and Fisher's exact test were used. Significant variables were subsequently included in a binary logistic regression analysis to determine independent risk factors for resistance to each antibiotic. The statistical software package SPSS 20.0 for Windows (SPSS, Chicago, IL, USA) was used.

Ethical considerations

The study was approved by the ethics committee of our Hospital and the Faculty of Medicine, and was performed in

accordance with all standards of good clinical practice. Signed informed consent was obtained from each patient.

Results

A total of 180 patients (131 female; mean age 43.4 ± 13.5 years; Group I 103; Group II 77) were included. Demographic and clinical characteristics are presented in Table 1. For the 77 previously treated patients the median number of eradication attempts was one (range one to six) with the following drugs: amoxicillin, 77 (100%); clarithromycin, 73 (94.8%); nitroimidazoles, 24 (31.2%); levofloxacin, 12 (15.6%).

Endoscopy with biopsies was successfully and safely performed in all patients. We obtained 360 *H. pylori* isolates (antrum, 180; corpus, 180) and in eight patients there were differences between isolates from the antrum and the corpus, concerning mutations for *23S rRNA* genes and resistance to clarithromycin. We considered only one isolate for each patient, always selecting the one with resistance.

TABLE 1. Demographic and clinical characteristics of the patients

	Group I *n* = 103	Group II *n* = 77	p value
Mean age (years)	41.6 ± 12.9 (range 19–77)	45.8 ± 14.1 (range 18–85)	0.039
Gender (%)			
Male	30 (29.1)	19 (24.7)	0.612
Female	73 (70.9)	58 (75.3)	
Ethnic background (%)			
European	99 (96.1)	76 (98.7)	0.394
African	4 (3.9)	1 (1.3)	
Education level (%)			
Level 1	30 (29.1)	33 (42.9)	0.06
Level 2	73 (70.9)	44 (57.1)	
Residence (%)			
Rural	55 (53.4)	47 (61)	0.362
Urban	48 (46.6)	30 (39)	
Indication(s) for *Helicobacter pylori* eradication (%)			
Non-ulcer dyspepsia	63 (61.2)	53 (68.8)	0.346
GERD/Chronic therapy with PPI	24 (23.3)	22 (28.6)	0.491
First-degree relatives with gastric cancer	14 (13.6)	15 (19.5)	0.311
Iron-deficient anaemia	25 (24.3)	10 (13)	0.086
Peptic ulcer	9 (8.7)	7 (9.1)	1
BMI (kg/m^2)	24.6 ± 4.2 (range 17.1–37.8)	25.2 ± 4.1 (range 17.1–33.8)	0.371
Smoking (%)	13 (12.6)	17 (22.1)	0.108
Alcohol consumption (%)	21 (20.4)	19 (24.7)	0.587
Olive oil consumption <1 dL/week (%)	50 (48.5)	40 (51.9)	0.763
History of frequent infections (%)	20 (19.4)	18 (23.4)	0.581
Antibiotic consumption in the last 12 months (%)	28 (27.2)	60 (77.9)	<0.0001
Family history of gastric diseases (%)	49 (47.6)	42 (54.5)	0.370
Family history of *H. pylori* infection (%)	10 (9.7)	9 (11.7)	0.807

BMI, Body Mass Index; GERD, gastro-oesophageal reflux disease; PPI, proton-pump inhibitors; Level 1, No education/Primary grades (Primary school); Level 2, Secondary grades (High-school)/University level.

TABLE 2. Pattern of resistance for the 180 isolates (one per patient)

Resistance	Total (*n* = 180) (%)	Group I (*n* = 103) (%)	Group II (*n* = 77) (%)	p value
Amoxicillin	1 (0.6)	1 (1)	0 (0)	1
Clarithromycin	90 (50)	22 (21.4)	68 (88.3)	<0.0001
Tetracycline	1 (0.6)	0 (0)	1 (1.3)	0.428
Metronidazole	62 (34.4)	30 (29.1)	32 (41.6)	0.113
Levofloxacin	61 (33.9)	27 (26.2)	34 (44.2)	0.017
No resistance	52 (28.9)	47 (45.6)	5 (6.4)	<0.0001
Single resistance	61 (33.9)	36 (35)	25 (32.5)	0.753
Dual resistance	49 (27.2)	17 (16.5)	32 (41.6)	<0.0001
Triple resistance	17 (9.4)	3 (2.9)	14 (18.2)	0.001
Quadruple resistance	1 (0.6)	0 (0)	1 (1.3)	0.428
Multidrug-resistant	67 (37.2)	20 (19.4)	47 (61)	<0.0001
Clarithromycin + Metronidazole	36 (20)	6 (5.8)	30 (39)	<0.0001
Clarithromycin + Levofloxacin	41 (22.8)	9 (8.7)	32 (41.6)	<0.0001
Metronidazole + Levofloxacin	26 (14.4)	11 (10.7)	15 (19.5)	0.133

Global, primary and secondary resistance rates are presented in Table 2. The only strain resistant to amoxicillin had a MIC level of 2 mg/L.

Genotyping revealed mutations in *23S rRNA* in 86 isolates (47.8%), in the QRDR region of the *gyrA* gene in 50 (27.8%) isolates, and mutations in *16S rRNA* in four isolates (2.2%), two $AGA_{926–928}$-GGA, one $AGA_{926–928}$-GTA and another $AGA_{926–928}$-TTC. For the first two, the MIC level was 0.016 mg/L, for the third it was 0.094 mg/L and for the last one it was 32 mg/L. So, only this isolate had *in vitro* resistance to tetracycline. For technical reasons it was not possible to study the *gyrA* gene in three isolates with resistance to levofloxacin. Mutations in *23S rRNA* and *gyrA* genes were detected in 86 (95.6%) and 50 (86.2%) of the isolates with *in vitro* resistance to clarithromycin and levofloxacin, respectively.

The *cagA* gene was detected in 63 strains (35%), the *vacA* s1m1 in 34 (18.9%), s1m2 in 48 (26.7%), s2m1 in 3 (1.7%) and s2m2 in 95 (52.7%). The gene *iceA1* was positive in 71 (39.4%) isolates and *babA2* in 21 (11.7%). The distribution of *vacA* genotypes according to *cagA* status is presented in Table 3.

Clinical characteristics and bacterial genotypes associated with resistance to clarithromycin, metronidazole and levofloxacin are resumed in Table 4. There were no significant differences for ethnic background, place of residence, non-ulcer dyspepsia, need for chronic therapy with PPI, iron-defi-

TABLE 3. *vacA* genotype distribution according to *cagA* status

Genotype	*cagA*-positive (*n* = 63) (%)	*cagA*-negative (*n* = 117) (%)	p value
s1m1	28 (44.4)	6 (5.1)	<0.0001
s1m2	22 (34.9)	26 (22.2)	0.076
s2m1	2 (3.2)	1 (1.7)	1
s2m2	11 (17.5)	84 (71.8)	<0.0001

TABLE 4. Clinical characteristics and bacterial genotypes associated with antibiotic resistance (univariate analysis)

	Clarithromycin			Metronidazole			Levofloxacin		
	Resistant *n* = 90	Susceptible *n* = 90	p value OR (95% CI)	Resistant *n* = 62	Susceptible *n* = 118	p value OR (95% CI)	Resistant *n* = 61	Susceptible *n* = 119	p value OR (95% CI)
Female	73 (81.1%)	58 (64.4%)	**0.019 2.37 (1.20–4.69)**	53 (85.5%)	78 (66.1%)	0.008 3.02 (1.35–6.76)	50 (82%)	81 (68.1%)	0.047 2.13 (1.01–4.55)
Age	45.9 ± 13.7	40.9 ± 12.9	**0.013**	44.5 ± 13.7	42.9 ± 13.5	0.445	46.7 ± 13.9	41.7 ± 13.1	0.02
>40 years	63 (70%)	46 (51.1%)	**0.014 2.23 (1.21–4.12)**	40 (64.5%)	69 (58.5%)	0.521	40 (65.6%)	69 (58%)	0.339
No or Primary level of education	41 (45.6%)	22 (24.4%)	**0.005 2.59 (1.37–4.88)**	24 (38.7%)	39 (33.1%)	0.511	29 (47.5%)	34 (28.6%)	0.014 2.27 (1.19–4.30)
First-degree relatives with gastric cancer	16 (17.8%)	13 (14.4%)	0.686	9 (14.5%)	20 (16.9%)	0.832	16 (26.2%)	13 (10.9%)	0.011 2.90 (1.29–6.54)
History of frequent infections	24 (26.7%)	14 (15.6%)	0.099	14 (22.6%)	24 (20.3%)	0.848	20 (32.8%)	18 (15.1%)	0.011 2.74 (1.32–5.68)
Antibiotics in the last 12 months	65 (72.2%)	23 (25.6%)	**<0.0001 7.58 (3.90–14.71)**	34 (54.8%)	54 (45.8%)	0.274	35 (57.4%)	53 (44.5%)	0.117
cagA positive	20 (22.2%)	43 (47.8%)	**<0.0001 0.31 (0.16–0.60)**	17 (27.4%)	46 (39%)	0.068	21 (34.4%)	42 (35.3%)	1
vacA s1m1	8 (8.9%)	26 (28.9%)	**0.001 0.24 (0.10–0.57)**	9 (14.5%)	25 (21.2%)	0.321	10 (16.4%)	24 (20.2%)	0.688
vacA s2m2	59 (65.6%)	36 (40%)	**0.001 2.86 (1.56–5.24)**	40 (64.5%)	55 (46.6%)	0.028 2.08 (1.11–3.92)	31 (50.8%)	64 (53.8%)	0.754
babA2	6 (6.7%)	15 (16.7%)	**0.037 0.36 (0.13–0.97)**	5 (8.1%)	16 (13.6%)	0.335	6 (9.8%)	15 (12.6%)	0.634

Age (years); BMI, body mass index (kg/m^2). Odds Ratios are presented for significant variables only.

cient anaemia, peptic ulcer, body mass index, tobacco/alcohol/olive oil consumption, *vacA* s1m2, *vacA* s2m1 and *iceA1* genotypes. Variables from Table 4 were subsequently included in a multivariate analysis that revealed the following positive relations: clarithromycin resistance with female sex (OR = 3.28; 95% CI 1.07–10.05) and previous, failed eradication treatments (OR = 31.32; 95% CI 10.06–98.48); metronidazole resistance with female sex (OR = 2.78; 95% CI 1.23–6.26); levofloxacin resistance with history of frequent infections (OR = 2.61; 95% CI 1.19–5.71); first-degree relatives with gastric carcinoma (OR = 2.63; 95% CI 1.10–6.26); and low education levels (OR = 2.43; 95% CI 1.21–4.85).

Discussion

The effectiveness of most commonly recommended *H. pylori* eradication treatments has declined to unacceptablly low levels [2]. So, it is important to periodically determine the resistance patterns in a certain region to establish what are the best therapy combinations to eliminate this bacterium.

In Portugal, *H. pylori* resistance rates can be as high as 39.4% for clarithromycin, 33.3% for metronidazole and 26.3% for levofloxacin. Resistance to amoxicillin and tetracycline are very rare [5,7,10,21,22]. Data about secondary resistance are scarce although Cabrita *et al.* [21] reported, in 2000, values of 75% and 47% for metronidazole and clarithromycin, respectively. To the best of our knowledge these data were obtained in the metropolitan area of Lisbon and there is no information about this problem in the central region of Portugal. With the present study we established that in this part of the country primary resistance rates to metronidazole and levofloxacin are comparable to the ones published recently by Megraud *et al.* [5]. Primary resistance to clarithromycin is lower than reported in the same study (21.4% versus 31.5%) but it surpasses the value established as the limit to allow empiric triple therapy with this antibiotic [1]. Resistance rates were higher in patients with failed eradications and, in this group, are similar to the ones reported in the literature, ranging from 40–70% for metronidazole, 50–85% for clarithromycin and multidrug-resistant in 51–90% [6,23–25]. This is very problematic because, in Portugal, many anti-*H. pylori* drugs such as bismuth, tetracycline, furazolidone, nitazoxanide and rifabutin are unavailable or cannot be used. It is of crucial importance to choose the most effective regimen on each occasion because failures are potentially untreatable.

Only the strain with the triple base-pair substitution $AGA_{926–928}$-TTC had tetracycline resistance and, in contrast to what is reported in the literature, the isolate with $AGA_{926–928}$-GTA had MIC levels within the susceptible range [17]. We also isolated only one strain resistant to amoxicillin, confirming the high levels of *H. pylori* susceptibility to this antibiotic in all circumstances.

The secondary resistance rate to clarithromycin was higher than previously reported for Portugal but similar to results from other European countries [21,26]. Mutations in *23S rRNA* were identified in 95.6% of isolates with phenotypic resistance. We only studied the three classic mutations described in the literature that are responsible for >90% of clarithromycin-resistant clinical strains but there are several other point mutations in *23S rRNA*, some of them associated with low resistance levels [4,27]. So, it is possible that the four strains with *in vitro* resistance and no genotypic alterations could have any of such mutations or there could be other responsible mechanisms such as the efflux pump system [27].

Resistance rates to metronidazole were high but did not increase significantly in patients with unsuccessful eradication attempts. This can be explained because failed treatments with nitroimidazoles do not systematically determine resistance. On the other hand, we should not forget that metronidazole susceptibility testing by Etest can overestimate resistance by 10 to 20% in comparison with the agar dilution method [12]. We tried to overcome this problem by using a control strain and repeating the test by a different microbiologist.

Primary and secondary resistance rates to levofloxacin are worrisome. The high values detected for primary resistance are probably explained because fluoroquinolones are widely employed for the treatment of other common bacterial infections and our country has high levels of outpatient antibiotic consumption. It is possible that loss of susceptibility was determined by pre-treatment *H. pylori* exposure to inadequate levels of antimicrobials [5,26,28]. This is a limitation of our work because, for group II, we had no data about antibiotic susceptibility before eradication treatments.

High macrolide and fluoroquinolone consumption for minor respiratory and urinary infections can also explain why resistance to clarithromycin and levofloxacin is higher in older individuals, given the cumulative probability of exposure to such drugs. Megraud *et al.* [5] also found that older age was a risk factor for levofloxacin resistance.

Presence of *gyrA* mutations was associated with resistance to levofloxacin but not all isolates with lack of *in vitro* susceptibility had genotypic alterations. This can occur because we did not test all possible mutations. It would be important to sequence QRDR in genes *gyrA* and *gyrB* to confirm these possible associations.

Women more frequently have *H. pylori* strains resistant to macrolides, fluoroquinolones and nitroimidazoles. This finding

is consistent with other studies and is determined by the increased use of antimicrobials in women to treat gynaecological and urinary infections.

Patients with lower educational levels had increased *H. pylori* resistance to clarithromycin and levofloxacin. We can speculate that they had lower socioeconomic status and eventually more unnecessary exposure to antibiotics. However, we have no data to explain these differences and a more consistent epidemiological approach would be necessary to confirm these results.

Our data did not support a correlation of clinical presentation with antibiotic resistance but one major limitation of our study is the high number of patients with non-ulcer dyspepsia [5]. We found no plausible explanation for the association between resistance to levofloxacin and first-degree relatives with gastric cancer and this must be confirmed in other studies.

Our study revealed the possibility of mixed infection by antibiotic hetero-resistant *H. pylori*. This was already reported in the literature and a resistant strain in any site of the stomach is enough to determine treatment failure [23,29]. For that reason, we suggest that in *H. pylori* antibiotic susceptibility testing it is important to obtain samples from at least two different locations and these biological materials must be processed independently.

The genes *cagA* and *vacA* have been identified as the main *H. pylori* virulence factors. There are reports of a strong association between eradication success, antibiotic susceptibility and these virulence factors, mainly for clarithromycin [4,30]. The present study also indicated that s2m2 strains, which are mostly *cagA* negative, seem to be more resistant to clarithromycin and metronidazole than strains with the s1m1 and s1m2 combinations.

Conclusions

Primary and secondary resistance rates to clarithromycin, metronidazole and levofloxacin are very high in the central region of Portugal and, at least for clarithromycin, are associated with previous eradication treatments. In contrast, resistance rates to amoxicillin and tetracycline are negligible, even after systematic use of amoxicillin.

In vitro resistance to clarithromycin and levofloxacin can be predicted in most cases by the presence of specific mutations. Molecular methods for determining resistance in biopsy or stool specimens could be very useful in clinical practice, potentially avoiding expensive and time-consuming cultures.

Multidrug-resistant isolates are common and probably, the quadruple concomitant or quadruple hybrid therapies are the best options for first-line treatment in Portugal.

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Author Contributions

The involvement of each author was as follows: study concept and design: NA, MMD, JMR, CL, OC, MAC, CM; acquisition of data: NA, MMD, JMR, CL, OC, MAC, CM, AF, CC; analysis and interpretation of data: NA, JMR, AF; drafting of the manuscript: NA, JMR, AF; critical revision of the manuscript for important intellectual content: NA, MMD, JMR, CL, OC, MAC, CM, AF, CS; statistical analysis: NA, JMR, AF; administrative, technical, or material support: NA, MMD, JMR, CC; study supervision: NA, JMR, CS.

Transparency Declaration

There are no conflicts of interest to disclose.

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