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Updating Primary Antibiotic Resistance in *Helicobacter pylori* Strains Isolated in Italy

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ABSTRACT

Background & Aims: Bacterial resistance toward the most used antibiotics is increasing in *Helicobacter pylori* (*H. pylori*) strains worldwide. The emergence of multidrug resistance significantly affects the efficacy of standard therapy regimens. Therefore, this prospective study has updated the prevalence rates of primary antibiotic resistance in *H. pylori* strains isolated in routine practice.

Methods: *H. pylori* isolates obtained from patients consecutively observed in a single center were tested for primary resistance by using the E-test method. The minimum inhibitory concentration (MIC) breakpoints to define resistance to clarithromycin, metronidazole, and levofloxacin were, respectively, greater than 0. 25 mg/L, 8 mg/L, and 1 mg/L, according to updated EUCAST recommendations. The trend of antibiotic prevalence, either single or combined, during 2020–2023 was assessed.

Results: A total of 789 patients meeting inclusion criteria were diagnosed with *H. pylori* infection, but bacterial strains were overall recovered in 632 (80.1%) cases. At bacterial culture, primary resistance rate was 36.7% for clarithromycin, 32.8% for metronidazole, and 22.1% for levofloxacin, whilst dual clarithromycin-metronidazole resistance rate was detected in 17.4%, and triple resistance in 9%.

Conclusions: Our data found that primary resistance towards both clarithromycin and metronidazole, as well as dual resistance, is substantially stable in Italy whilst the prevalence of levofloxacin resistance seems to be decreasing in our geographic area.

Key words: *H. pylori* – primary antibiotic resistance – clarithromycin – metronidazole – levofloxacin –culture.

Abbreviations: BMI: body mass index; CI: confidence interval; *H. pylori: Helicobacter pylor*i; MIC: minimum inhibitory concentration; NUD: non-ulcer dyspepsia; PPI: proton pump inhibitor; PUD: peptic ulcer disease.

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INTRODUCTION

Helicobacter pylori (*H. pylori*) infection causes different upper gastrointestinal diseases, including non-ulcer dyspepsia, peptic ulcer, mucosa-assisted lymphoid tissue (MALT) lymphoma, and gastric cancer [1-4]. Regrettably, curing this infection is still challenging, due to the availability of only a few active antibiotics and the increasing rate of primary antibiotic resistance towards different drugs, so that eradicationtherapy has become a growing problem [5, 6]. In 2017, the World Health Organization published a list of antibiotic-resistant 'priority pathogens', a catalogue of bacteria that poses the greatest threat to human health, and clarithromycin-resistant *H. pylori* strains were classified as high-priority bacteria [7].

The rates of *H. pylori* antibiotic resistance towards clarithromycin, metronidazole, and levofloxacin - which are the most frequently used drugs in the current eradication regimens - has increased in the last decades [8-10], whilst amoxicillin, tetracycline, and rifabutin resistance rates still remain low. The prevalence of primary antibiotic resistance in *H. pylori* isolates may change over time, but data on this clinically relevant issue are only scantly reported in literature. Therefore, we designed this prospective study to update the prevalence rates of primary antibiotic resistance in *H. pylori* strains isolated in routine practice, and to search for potentially involved factors.

METHODS

Patients

The present data were collected in a single Endoscopic Unit in Bologna, Italy. Data refer to *H. pylori*-positive, naïve, consecutive patients referred by their primary care physicians to perform an esophagogastroduodenoscopy, between January 2020 and June 2023. Exclusion criteria included: a) previous *H. pylori* eradication; b) use of proton pump inhibitors (PPIs) or antibiotics in the last 2 weeks before the endoscopy; c) gastric surgery; d) contraindication to take gastric biopsies; e) age: < 18 years. For each patient, information regarding gender, age, body mass index (BMI), smoking habit (at least one cigarette/day), alcohol intake (at least one drink/day), and country of birth were registered. All participants signed informed consent to undergo endoscopic procedure and for anonymous use of their clinical data for scientific purposes. The Ethical Committee approved the present study (code 47/2012/U/OSS).

Esophagogastroduodenoscopy

Standard endoscopic examination was performed, and eight biopsy specimens were taken on gastric mucosa. In detail, five biopsy samples were taken according to the standard gastric sampling (two from the antrum, one from the incisura angularis, two from the body) and used for histological examination with hematoxylin-eosin staining, and modified Giemsa staining when needed, for both gastritis assessment and *H. pylori* detection. Two additional antral biopsies were taken to perform rapid urease test (Biohit ultrafast UFT-300 kit, Biohit Healthcare, Milan, Italy) and the other for bacterial culture. *Helicobacter pylori* infection was diagnosed when both histological examination and rapid urease test were positive and/or culture identified specific bacterial strains. Based on endoscopic findings, patients with ulcer (ulceration 5 mm in diameter) or mucosal erosions in the stomach or duodenum were grouped as peptic ulcer disease (PUD) patients, whilst when no macroscopic lesions were detected, patients were considered as non-ulcer dyspepsia (NUD) cases.

Antibiotic Susceptibility Testing

One antral biopsy was immediately seeded on commercial Pylori Agar selective medium (BioMérieux Italia, Florence, Italy). Plates were incubated in microaerobiosis, obtained in jars by Campygen gaspack (Oxoid, Milan, Italy), at 37°C for 3 to 5 days. Colonies resembling *H. pylori* were identified by oxidase, catalase and urease tests. *H. pylori* colonies were suspended in sterile saline at the McFarland 3 opacity standard, approximately 10^9 colony forming units (CFU)/mL to perform the E-Test (BioMérieux Italia, Florence, Italy). A total of four plates of Mueller Hinton Fastidious agar (Oxoid, Milan, Italy) for each *H. pylori* strains were seeded in three directions with a swab dipped in the bacterial suspension to produce a coating growth. Three E-Test strips (clarithromycin 0.016-256 μg/mL, metronidazole 0.016-256 μg/mL, metronidazole 0.016-256 μg/mL, and levofloxacin 0.008-32 μg/mL) were each placed on one plate, whilst the fourth plate was used as a growth control. The plates were incubated in jars with gaspack for microareobiosis (Campygen, Oxoid, Milan, Italy) at 37°C for 72 hours. The minimum inhibitory concentration (MIC) breakpoints to define resistance to clarithromycin, metronidazole, and levofloxacin were, respectively, greater than 0.25 mg/L, 8 mg/L, and 1 mg/L, according to updated EUCAST recommendations [11]. From January 2015 to December 2022, EUCAST stipulated for clarithromycin that MICs >0.25 and ≤0.5 should be considered "susceptible, increased exposure". Although they were reported with this wording, for therapeutic purposes, these strains were still considered resistant, and are grouped with resistant strains in our study.

Statistical Analysis

The prevalences and their 95% confidence intervals (CI) for the tested antibiotics were calculated, and comparisons among groups were performed using the Chi-square test (Yates correction, if appropriate). Considered factors potentially related to primary resistance were a) age (>50 years or ≤50 years); b) gender; c) BMI (>25 or ≤25); d) smoking habit (at least one cigarette per day); e) alcohol consumption (at least one drink per day); and f) endoscopic findings (PUD or NUD). A p level lower than 0.05 was considered significant. Statistical analyses were performed with MedCalc19.1.

RESULTS

A total of 789 patients meeting the inclusion criteria were diagnosed with *H. pylori* infection, but bacterial strains were overall recovered in 632 (80.1%) cases (Fig. 1). The mean age of these patients was 47 years (range: 18-89); there were 240 (38%) males, 118 (18.7%) smokers, 64 (10.1%) drinkers, and 137 foreigners (29 from Africa, 84 from East Europe, 9 from South America, 15 from Asia). At endoscopic examination, PUD was diagnosed in 80 (12.7%), gastric lymphoma in 6 (0.9%), gastric cancer in 2 (0.3%), whilst no lesion was detected in 545 (86.2%) cases.

At antibiotic susceptibility testing, primary resistance rate was 36.7% (n=232; 95%CI: 33.0-40.5) for clarithromycin, 32.8% (n=207; 95%CI: 29.2-36.5) for metronidazole, and 22.1% (n=140; 95%CI:19.1-25.5) for levofloxacin. Dual clarithromycin-metronidazole resistance rate was detected in

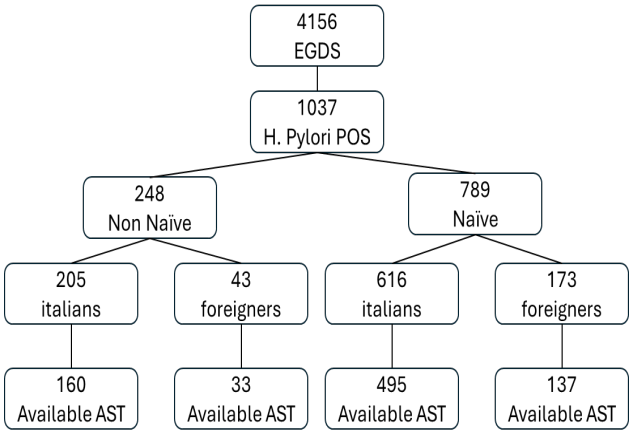

Fig. 1. Flow chart of the enrolled population. AST: antibiotic susceptibility test. EGDS: esophagogastroduodenoscopy; POS: positive; AST: antibiotic susceptibility test; naïve: patients who have never undergone eradication therapy; non-naïve: patients who have already undergone one or more eradication therapies.

8.4% (n=53; 95%CI: 6.4-10.8), and triple resistance in 9.0% (n=57; 95%CI: 7.0-11.5). Strains with double clarithromycin-metronidazole resistance, regardless of levofloxacin status, were 17.4% (n=110, 95%CI: 14.7-20.6) (Table I). The pattern of bacterial resistance observed in Italian and foreign patients was provided in Table II. The overall prevalence of resistant strains (at least one antibiotic) was significantly higher in foreigner than Italian patients (96/137, 70.1% vs 261/495, 52.7%; p<0.001). In detail, as shown in Table II, in foreign there was a higher frequency of dual and triple antibiotic resistances.

Table I. Pattern of primary bacterial resistance in *H. pylori* isolates

Pattern	N	% (95% CI)
ClaR, MtzR, LevR	57	9 (7-11.5)
ClaR, MtzR, LevS	53	8.4 (6.1-10.3)
ClaR, MtzS, LevR	26	4.1 (2.8-6)
ClaR, MtzS, LevS	96	15.2 (12.6-18.2)
ClaS, MtzR, LevR	29	4.6 (3.2-6.5)
ClaS, MtzR, LevS	68	10.8 (8.6-13.4)
ClaS, MtzS, LevR	28	4.4 (3.1-6.3)
ClaS, MtzS, LevS	275	43.5 (39.7-47.4)
ClaR tot	232	36.7 (33.0-40.5)
MtzR tot	207	32.8 (29.2-36.5)
LevR tot	140	22.1 (19.1-25.5)

For abbreviations see Table I.

Table II. Distribution of primary antibiotic resistance between Italian and foreigner patients

	Italians (N = 495)	Foreigners (N = 137)	p
ClaR, MtzR, LevR	37 (7.5%)	20 (14.6%)	0.01
ClaR, MtzR, LevS	35 (7.1%)	18 (13.1%)	0.023
ClaR, MtzS, LevR	18 (3.6%)	8 (5.8%)	0.3
ClaR, MtzS, LevS	79 (15.9%)	17 (12.4%)	0.3
ClaS, MtzR, LevR	18 (3.6%)	11 (8%)	0.03
ClaS, MtzR, LevS	51 (10.3%)	17 (12.4%)	0.5
ClaS, MtzS, LevR	23 (4.6%)	5 (3.6%)	0.6
ClaS, MtzS, LevS	234 (47.3%)	41 (29.9%)	<0.001
ClaR tot	169 (34.1%)	63 (46.0%)	0.01
MtzR tot	141 (28.5%)	66 (48.2%)	<0.001
LevR tot	96 (19.4%)	44 (32.1%)	0.001

For abbreviations see Table I.

When considering data of Italian patients, female gender was significantly associated with the presence of both single metronidazole resistance (OR=1.56, p=0.035) and dual clarithromycin/metronidazole resistance (OR=1.99; p=0.017), whilst age over 50 years tended to be associated with triple resistance (OR=2.21, p=0.052), approaching the statistical significance (Table III).

Table III. Factors associated with antibiotic resistance

Factor	Resistance pattern	OR (95% CI)	p
Gender:	Cla R	1.15 (0.78-1.70)	0.42
Female vs male	Mtz R	1.56 (1.03-2.37)	0.035
	Lev R	1.34 (0.83-2.15)	0.21
	ClaR, MtzR	1.99 (1.08-3.30)	0.017
	ClaR, MtzR, LevR	0.88 (0.44-1.74)	0.71
Age:	Cla R	1.00 (0.68-1.47)	0.97
>50 vs <50 years	Mtz R	1.29 (0.75-1.69)	0.55
	Lev R	1.20 (0.75-1.92)	0.43
	ClaR, MtzR	0.84 (0.50-1.40)	0.5
	ClaR, MtzR, LevR	2.21 (0.99-4.96)	0.052
BMI:	Cla R	0.95 (0.65-1.39)	0.81
>25 vs <25	Mtz R	0.90 (0.60-1.35)	0.62
	Lev R	1.05 (0.66-1.66)	0.82
	ClaR, MtzR	1.16 (0.69-1.93)	0.56
	ClaR, MtzR, LevR	1.23 (0.62-2.42)	0.54
Smoking habit:	Cla R	1.13 (0.71-1.80)	0.59
Yes vs not	Mtz R	1.11 (0.68-1.81)	0.68
	Lev R	1.30 (0.76-2.23)	0.33
	ClaR, MtzR	0.71 (0.36-1.42)	0.34
	ClaR, MtzR, LevR	1.16 (0.51-2.62)	0.72
Alcohol:	Cla R	1.27 (0.70-2.31)	0.42
Yes vs not	Mtz R	0.94 (0.49-1.80)	0.86
	Lev R	1.01 (0.49-2.10)	0.96
	ClaR, MtzR	1.29 (0.60-2.79)	0.58
	ClaR, MtzR, LevR	0.06 (0.36-3.12)	0.91
Peptic ulcer disease:	Cla R	0.81 (0.45-1.44)	0.47
Yes vs not	Mtz R	1.41 (0.81-2.46)	0.22
	Lev R	1.09 (0.56-2.11)	0.79
	ClaR, MtzR	0.71 (0.31-1.61)	0.41
	ClaR, MtzR, LevR	1.36 (0.24-3.40)	0.51

For abbreviations see Table I.

DISCUSSION

Helicobacter pylori infection remains a largely prevalent infection worldwide [12-15]; the rise of antibiotic-resistant strains has led not only to a decline in treatment efficacy, but also has led to the search for new diagnostic tools, such as molecular approaches [16]. Indeed, the culture test represents a methodology with specificity close to 100% and sensitivity between 85-95%, but it is burdened by several critical issues represented by sample transport and storage, and inherent difficulties in isolating the bacteria in culture. In fact, *H. pylori* is defined as a "fastidious" i.e., nutrient-demanding and slow-growing bacterium. It requires complex culture media. Often these media are supplemented with blood or serum. These supplements can serve as additional sources of nutrients and possibly also protect against the toxic effects of long-chain fatty acids [17]. The latter function can also be performed by more defined media supplements, such as β-cyclodextrins or IsoVitaleX, or by the use of activated charcoal [18].

The decreasing treatment efficacy mostly depend on the increased prevalence of primary bacterial resistance towards the most used antibiotics, namely clarithromycin and metronidazole [8, 9, 19]. These drugs are currently included in all non-bismuth quadruple therapies (concomitant, hybrid and sequential regimens) and, partially, also in the bismuth-based treatments. Moreover, levofloxacin is an antibiotic employed in different rescue therapies [20, 21]. The presence of primary antibiotic resistance towards a specific molecule decreases the efficacy of those therapy containing the same drug, and the therapies success further decreases when dual resistance is present [9, 22]. Indeed, current guidelines suggest that some

therapy regimens should be avoided in those areas where primary clarithromycin resistance is or dual clarithromycin-metronidazole resistance are >15% [21, 23]. Therefore, to monitor primary resistance in *H. pylori* isolates is of paramount relevance for therapeutic purposes. Data of the present study found that the prevalence of primary resistance in *H. pylori* isolates was >30% for both clarithromycin and metronidazole, with a rate of dual resistance towards these molecules >15%. Worth noting, when comparing Italian patients' data with those reported in the decade between 2009 and 2019 (data were divided in two five-years period) in the same geographic area, it emerges that clarithromycin and metronidazole resistance rates remained essentially unchanged [9]. By contrast, resistance rates to levofloxacin and clarithromycin/ metronidazole were significantly lower when compared to both five-year periods (levofloxacin actual period vs. first five-year period p=0.009; levofloxacin actual period vs. second five-year period: p<0.001; clarithromycin/metronidazole actual period vs. first five-year period: p=0.041, clarithromycin/metronidazole actual period vs. second five-year period 0.006). Triple resistance rates were not different from those reported in the first five-year period (p=0.075) but were significantly lower than those reported in the second five-year period (p=0.0041) [9].

Considering data from non-Italian patients we observed, at the level of overall resistance rates, no significant changes are observed compared to data published in 2020 regarding foreign naive patients enrolled at the same operating unit from 2009 to 2019 [9]. Only clarithromycin resistance rates, which remained stable among Italian patients, significantly increased in these patients (p=0.029) (metronidazole p=0.44, levofloxacin: p=0.86) The increase in clarithromycin resistance rates is mainly sustained by strains isolated from African patients, in which clarithromycin resistance rate increased from 37% to 62.07% (p=0.029). Analyzing the data (2009-2019 vs 2020-2023) divided by continent of origin, there is a significative increase in resistance also to clarithromycin/ metronidazole (p=0.019) only for African patients. The most updated review on bacterial resistance towards different antibiotics observed in *H. pylori* isolated worldwide showed that primary resistance rates to clarithromycin, metronidazole, and levofloxacin were >15% in all geographic areas, except primary clarithromycin resistance in the Americas (10%), and South-East Asia region (10%), as well as primary levofloxacin resistance in the European region (11%) [24].

Regarding predictive factors, we observed that the risk of both single metronidazole and dual clarithromycin-metronidazole resistances was 1.6-2.0-fold higher in females. The higher prevalence of primary resistance towards metronidazole in females is most likely linked to the diffuse use of nitroimidazoles for gynecological infections, as observed in other studies [25, 26].

Another relevant finding of present study was that the overall prevalence of antibiotic resistance in *H. pylori* strains isolated in foreigners is significantly higher than that in Italian patients, particularly for dual and triple resistances. A wider use of antibiotics due to a more frequent paediatric infections in developing countries may be hypothesised as potential involved factor for such an observation [27-29]. Therefore, it is foreseeable that such a phenomenon also occurs in other European countries. Since the presence of dual (or triple) resistance distinctly decreases the therapy success, the 2-fold higher prevalence of dual resistance should be taken into account by the clinicians when choosing the therapy regimen to treat *H. pylori* infection in foreigner patients. Finally, our data found that triple resistance tended to be higher in patients aged >50 years, and this finding should be taking into account when choosing first line and, particularly, rescue treatments.

CONCLUSIONS

Our data found that primary resistance towards both clarithromycin and metronidazole, as well as dual resistance, is substantially stable whilst the prevalence of levofloxacin resistance seems to be decreasing in *H. pylori* isolates in Italy during the study period.

Conflicts of interest: None to declare.

Authors' contribution: D.V. conceived and designed the study. D.V. selected the patients. M.P. and I.M.S. performed the microbiological analyses. I.M.S collected data. A.Z. performed the statistical analysis. I.M.S and M.V drafted the manuscript. D.V. is the guarantor of the paper. All the authors critically reviewed the manuscript and approved the final version of this manuscript.

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