ORIGINAL PAPERS

Primary Clarithromycin Resistance in *Helicobacter pylori*: the Multicentric Italian Clarithromycin Resistance Observational (MICRO) Study

Vincenzo De Francesco[1], Floriana Giorgio[1], Enzo Ierardi[1], Mariangela Zotti[1], Matteo Neri[2], Angelo Milano[2], Vincenzo Varasano[3], Francesco Luzza[4], Evelina Suraci[4], Riccardo Marmo[5], Armando Marone[5], Raffaele Manta[6], Vincenzo Giorgio Mirante[6], Marina de Matthaeis[7], Antonietta Pedroni[7], Gianpiero Manes[8], Stefano Pallotta[8], Paolo Usai[9], Mauro Liggi[9], Giovanni Gatto[10], Vittorio Peri[10], Rodolfo Sacco[11], Giampaolo Bresci[11], Fabio Monica[12], Cesare Hassan[13], Angelo Zullo[13]

1) Section of Gastroenterology, Department of Medical Sciences, University of Foggia, Foggia; 2) Department of Medicine and Aging Sciences, CESI Fundation, 'G. D'Annunzio' University, Chieti; 3) Section of Gastroenterology, 'Policoro' Hospital, Matera; 4) Department of Experimental and Clinical Medicine, 'Magna Graecia' University of Catanzaro; 5) Division of Gastroenterology, 'Curto' Hospital, Polla, Salerno; 6) Endoscopic Unit, 'Nuovo Ospedale Civile Sant'Agostino Estense' Baggiovara, Modena; 7) Department of Gastroenterology, General Hospital, Sanremo; 8) Department of Gastroenterology, 'L. Sacco' University Hospital, Milan; 9) Department of Internal Medical Sciences, University of Cagliari; 10) Gastroenterology and Digestive Endoscopy Unit 'Villa Sofia' Hospital, Palermo; 11) Gastroenterology and Metabolic Diseases, University of Pisa; 12) Endoscopy Unit, 'S. Bassiano' Hospital, Bassano del Grappa,Vicenza; 13) Gastroenterology and Digestive Endoscopy, 'Nuovo Regina Margherita' Hospital, Rome, Italy

Abstract

Background. Primary clarithromycin resistance markedly reduces *Helicobacter pylori* eradication rate following standard therapies. Prevalence of primary clarithromycin resistance in *H. pylori* is increasing, and three point mutations are mainly involved. **Aim**. To assess both the prevalence of primary clarithromycin resistance in Italy, and the distribution of the involved point mutations. **Methods.** Primary clarithromycin resistance was assessed by TaqMan real-time polymerase chain reaction on antral biopsies of 253 consecutive, *H. pylori* infected patients enrolled in 13 Italian centres between January and September 2010. **Results**. Primary clarithromycin resistance was detected in 25 (9.9%) patients, with prevalence values widely ranging from 0 to 25%. Clarithromycin resistance rate was higher in female as compared to male patients (13.4% vs. 5.3%, p=0.03), and it tended to be higher in non-ulcer dyspepsia than in peptic ulcer patients (10.6% vs. 6.9%, p=0.5), female patients with non-ulcer dyspepsia showing the highest value (15.4%). The A2143G point mutation was detected in 13 (52.0%) patients, the A2142G in 9 (34.6%), whilst a double point mutation (A2143G plus A2142G) in 3 (11.6%) cases. **Conclusions**. Primary clarithromycin resistance is highly variable in different Italian geographic areas. High resistance rates were observed in female and in dyspeptic patients. Among the three point mutations of clarithromycin resistance, the A2143G remains the most frequently observed.

Key words

Clarithromycin – resistance – *H. pylori* – real time PCR – point mutation.

Received: 20.05.2011 Accepted: 05.06.2011
J Gastrointestin Liver Dis
September 2011 Vol. 20 No 3, 235-239
Address for correspondence: Vincenzo De Francesco, M.D.
Section of Gastroenterology
Department of Medical Sciences
University of Foggia, Ospedali Riuniti
Viale L. Pinto 71100, Foggia, Italy
vdefrancesco@ospedaliriunitifoggia.it

Introduction

Prevalence of primary clarithromycin resistance is increasing in several developed countries according to recent studies [1, 2]. Therefore, to monitor primary antibiotic resistance would appear clinically relevant for *H. pylori* management in clinical practice, such a resistance being identified as the main factor for reducing the eradication rate [3]. Indeed, the use of a 'tailored-treatment' according to the clarithromycin resistance rate prevalence in each geographic area is proposed by current European guidelines [4].

Usually, *H. pylori* clarithromycin resistance assessment has been performed on bacterial cultures by specific antibiogram - including agar diffusion or dilution or E-tests. Recently, different culture-free polymerase chain reaction (PCR)-based clarithromycin resistance assays have been introduced allowing the detection of resistant strains with a very high accuracy (98%). By using such tools, it has been clarified that three main rRNA-point mutations (A2143G, A2142G, A2142C of domain V) are responsible for more than 90% of clarithromycin resistance cases, and different prevalence rates of such mutations have been reported worldwide [5-9]. Of note, these point mutations have been associated with both different levels of resistance and variable eradication rates [10-13]. In detail, presence of the A2143G point mutation has been shown to significantly affect *H. pylori* eradication rate [14, 15]. Consequently,

to investigate the distribution of each mutate genotype for clarithromycin resistance is important for *H. pylori* management in clinical practice.

We therefore designed the present, multicentric study in order to update the overall prevalence of primary clarithromycin resistance in Italy, and to assess the distribution of the involved point mutations.

Patients and Methods

Patients

Consecutive, >18 year old, dyspeptic patients referred for upper endoscopy in the participating centres between January and September 2010 were considered for enrolment. During endoscopy, biopsy specimens from antrum and gastric body were performed for rapid urease test and histological assessment. Two further antral biopsy specimens were collected for clarithromycin resistance assessment. *H. pylori* infection was considered present when bacteria were detected both at histology, jointly with an active chronic gastritis, and when rapid urease was positive. The study enrolled only Italian patients never previously treated for *H. pylori* infection. Patients who had taken proton-pump inhibitors or antibiotics during the previous 8 weeks before endoscopy were also excluded. Non-ulcer dyspepsia was defined as pain or discomfort centred in the upper abdomen without macroscopic lesions at endoscopy, whereas peptic ulcer was defined as a mucosal lesion >5 mm in diameter in either the gastric or duodenal mucosa. To participate in the present study, each centre was required to enrol 10-20 consecutive *H. pylori* positive patients. Informed consent to take two antral biopsy specimens for antibiotic susceptibility testing was obtained from each patient before endoscopy. The study protocol was approved by the Ethical Committee of the "Riuniti Hospitals" of Foggia.

Quantitative PCR

All biopsy specimens were referred to a single centre (Foggia). Paraffin embedded gastric biopsies were obtained and used to assess primary clarithromycin resistance by using TaqMan real-time PCR. The assessment was blinded, performed by a single operator. DNA was extracted from the same antral samples as used for histology by using a NucleoSpin Tissue kit (Macherey-Nagel GmbH&Co, Germany) applied on paraffin-embedded sections (at least five sections of 10μm), which are largely accepted to constitute a reliable substrate for DNA analysis similar to fresh material. The A2142C, A2142G and A2143G point mutations were investigated as previously described [16]. Briefly, real-time PCR was performed in 96-well plates using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Monza, Italy). The final reaction volume (25 μL) was analysed in triplicate (three samples for each patient) and all experiments were repeated twice. In detail, we used the same primers and probes for allelic discrimination of mutant genes which were provided in a previous study [16]. In our experience, the variability between duplicates and triplicates within the same run or different runs was usually between 0 and 2% [17].

Statistical analysis

Differences between groups were statistically evaluated by using the Student's t-test for unpaired data, Chi-squared test and Fisher's exact probability test as appropriate. The odds ratio with 95% confidence intervals was also calculated. Logistic multivariate analysis was performed in order to identify possible risk factors (age, gender, gastroduodenal disease) for antibiotic resistance. Differences were considered significant at 5% probability level. Statistical analysis was performed using a specific software (STATSOFT 7.1 program for Windows XP).

Results

Bacterial clarithromycin resistance was assessed on 253 consecutive, *H. pylori*-positive patients, collected in 13 different Italian centres (4 from Northern Italy; 3 from Central Italy; 6 from Southern Italy). Demographic and clinical characteristics of patients are provided in Table I. Overall, primary clarithromycin resistance was detected in 25 (9.9%; 95% CI = 6.2-13.5) patients, with prevalence values widely ranging from 0 to 25%. Clarithromycin resistant strains were detected in 5 (7.9%) out of 63 patients from Northern Italy, in 7 (10.1%) out of 69 patients from Central Italy, and in 13 (10.7%) out of 121 patients from Southern Italy, without a statistically significant difference among the 3 geographic areas.

At univariate analysis, a distinctly higher prevalence of clarithromycin resistance was observed in female (13.4%) as compared to male (5.3%) patients, the difference being statistically significant (OR: 2.7; 95% CI: 1.2 -7.1; p=0.03). Similarly, clarithromycin resistance observed in non-ulcer dyspepsia (10.6%) patients was twice as much compared to those in peptic ulcer (6.9%) patients, although the difference failed to reach a statistical significance.

Of note, among non-ulcer dyspepsia patients, prevalence of clarithromycin resistance was higher in females (19 out of 123; 15.4%) as compared to males (3 out of 86; 3.4%), the difference being statistically significant (OR 5.0; 95% CI: 1.4 - 17.6; p <0.01). Conversely, in the peptic ulcer group, clarithromycin resistance tended to be higher in male (3 out of 26; 11.5%) than in female (0 out of 18; 0%) patients (OR: 5.5, 95% CI: 0.2 - 11.3; p=0.25).

Overall, the mean age of patients infected with a clarithromycin resistant strain did not significantly differ from that of patients harbouring a susceptible *H. pylori* strain (48.6 ± 13.2 years vs 51.8 ±15.9; p=0.3). Similarly, clarithromycin resistance rate did not differ between young (<45 years) and old (≥45 years) patients, being present in 8 (9.0%) out of 88 and in 17 (10.3%) out of 165 patients, respectively. As shown in Table II, the multivariate analysis failed to identify an independent risk factor for primary clarithromycin resistance.

As far as genotypic pattern of resistance is concerned, the A2143G point mutation was detected in 13 (52.0%) patients, the A2142G in 9 (34.6%), whilst a double point mutation (A2143G plus A2142G) was present in the remaining 3 (11.6%) cases. Therefore, the A2143G (single or combined) was the most prevalent point mutation accounting for a total of 16 (64.0%) clarithromycin-resistant bacterial isolates. The A2142C mutate genotype is absent in the sample study.

Discussion

Clarithromycin still remains the key antibiotic for *H.*

Table I. Clinical characteristics of patients and clarithromycin resistance.

	No of patients	Sex (Male/Female)	Age (Mean ± SD)	Disease (NUD/PUD)*	Resistance N (%)
Northern Italy					
Lombardia	22	8/14	58.9±16.4	14/8	3 (13.6%)
Liguria	15	7/8	56.5±13.5	14/1	1 (6.7%)
Veneto	16	7/9	48.5±15.1	16/0	1 (6.3%)
Emilia Romagna	10	4/6	47.5±16.0	8/2	0 (0.0%)
Central Italy					
Toscana	20	8/12	57.9±14.7	19/1	3 (15%)
Abruzzo	28	10/18	48.9±12.1	24/4	2 (7.1%)
Lazio	21	11/10	55.2±11.9	14/7	2 (9.5%)
Southern Italy					
Calabria	20	10/10	48.8±15.4	14/6	2 (10.0%)
Campania	19	10/9	54.4±19.0	19/0	2 (10.5%)
Basilicata	20	11/9	49.0±15.1	16/4	0 (0.0%)
Puglia	21	6/15	52.8±15.4	19/2	4 (19.1%)
Sardinia	21	11/10	46.1±15.2	15/6	0 (0.0%)
Sicily	20	11/9	44.6±18.7	17/3	5 (25.0%)
Overall	253	114/139	51.5±15.6	209/44	25 (9.9%)

*NUD: non-ulcer dyspepsia; PUD: peptic ulcer

Table II. Clarithromycin resistance distribution according to clinical characteristics

	Susceptible trains	Resistant strains	P value (univariate analysis)	P value (multivariate analysis)
Disease				
PUD (44)	41 (93.1%)	3 (6.9%)	0.5	0.55
NUD (209)	187 (89.4%)	22 (10.6%)		
Sex				
M (112)	106 (94.7%)	6 (5.3%)	0.03	0.35
F (141)	122 (86.6%)	19 (13.4%)		
Age				
< 45 (88)	80 (91.0%)	8 (9.0%)	0.8	0.67
≥ 45 (165)	148 (89.7%)	17 (10.3%)		

PUD: Peptic Ulcer Disease. NUD: Non-Ulcer-Dyspepsia

pylori treatment, its in vitro activity being the most powerful as compared to the other available molecules [3]. Indeed, it is included in all standard therapy regimens, such as triple therapies, the sequential regimen or the concomitant therapy [4, 16, 19]. Unfortunately, primary clarithromycin resistance markedly affects *H. pylori* eradication rates following the current therapeutic regimens [3, 4]. Consequently, current European guidelines for *H. pylori* management suggest that standard 7-day triple therapy should be prolonged to 14 days where primary clarithromycin resistance is higher than 15-20% or shifted towards a quadruple therapy [4]. Therefore, antibiotic susceptibility monitoring is crucial to tailor first-line eradication therapy in different geographic areas. The present study is the first, multicentric investigation on *H. pylori* clarithromycin resistance performed in Italy, involving 13 out of 20 regions. Overall, data found that primary clarithromycin resistance is present in near 10% of bacterial isolates, and it was >15% in only 3 Italian regions, being as high as 25% in Sicily. Consequently, despite evident discrepancies from previous data of single or a few centres, the comparison with previous Italian data would indicate that primary clarithromycin resistance rate has not increased, but remains substantially stable in these last years [16, 20-24]. In detail, it has remained very stable (or reduced) in Puglia (23.3%) [16] Lazio (17.5%) [20], Abruzzo (7.0%) [21], Emilia Romagna (16.6%) [20] Sardinia (23.1%) [22] and Liguria (14.0%) [23] but it would appear increased in Veneto (1.8%) [24]. The discrepancy between present data and those previously reported in Italy regarding *H. pylori* primary clarithromycin resistance could be explained by the test used - i.e. PCR-based and culture-based methods [8]. In addition, the absence of the A2142C mutate genotype in the present study could be responsible – at least in part – for the lack of the increased resistance rate. It should be also

taken into account that the number of patients enrolled in each participating center is not high, so that comparison with previous data should be interpreted with caution.

Based on the overall resistance rate we observed, it could be suggested that an empirical choice of a clarithromycin-containing regimen - rather than a bacterial resistance-based therapy - could still remain the first-line therapeutic approach in clinical practice in Italy [21].

Worth noting was the fact that our data found that the prevalence rate of clarithromycin resistance was twice in non-ulcer dyspepsia as compared to peptic ulcer patients. Such a finding is consistent with previous observations [15, 25]. Such a phenomenon could play a role in the efficacy of standard eradication therapy observed according to gastroduodenal pathology, the cure rate being generally lower in non-ulcer dyspepsia patients [26-28]. Similarly, we found that clarithromycin resistance was higher in females as compared to males. A previous study showed that clarithromycin resistance increased 3-fold in females during a 15-year period whilst it remained stable in male patients [25]. Furthermore, it has been found that a *H. pylori* recurrence rate was distinctly higher in women with non-ulcer dyspepsia [29]. Such a phenomenon could depend on the higher prevalence of primary clarithromycin resistance in females with non ulcer dyspepsia, as we observed in the present study. Indeed, eradication therapy in those patients harbouring a clarithromycin resistant strain could lead to a bacterial clearance rather than a true eradication.

As far as the distribution of clarithromycin resistance point mutations is concerned, our study found the A2143G remained the most prevalent point mutation involved in *H. pylori* clarithromycin resistance, being present in more than 65% of resistant strains. Such a finding is in accordance with previous Italian studies and with observations performed in other Western countries [1, 15, 25, 30]. Of note, the A2143G point mutation has been found to be linked to high MIC values in vitro [10, 12] and its presence - different from the other two point mutations - markedly affects the eradication rate following standard triple therapy in vivo [13].

In conclusion, our study found a variable clarithromycin resistance prevalence in different Italian regions, while the overall resistance rate was lower than 10%. Of note, such a resistance was higher in females and non-ulcer dyspepsia patients, being particularly high in patients with both these characteristics. Among the three most common point mutations of clarithromycin resistance, the A2143G remains the most frequently detected.

Conflicts of interest

No funding required. No conflict of interest.

References

1. De Francesco V, Giorgio F, Hassan C, et al. Worldwide H. pylori antibiotic resistance: a systematic review. J Gastrointestin Liver Dis 2010; 19: 409-414.
2. Boyanova L, Mitov I. Geographic map and evolution of primary Helicobacter pylori resistance to antibacterial agents. Expert Rev Anti Infect Ther 2010; 8: 59-70.
3. Vakil N, Megraud F. Eradication therapy for Helicobacter pylori. Gastroenterology 2007; 133: 985-1001.
4. Malfertheiner P, Megraud F, O'Morain C, et al. Current concepts in the management of Helicobacter pylori infection: the Maastricht III Consensus Report. Gut 2007; 56: 772-781.
5. Owen RJ. Molecular testing for antibiotic resistance in Helicobacter pylori. Gut 2002; 50: 285-289.
6. Lascols C, Lamarque D, Costa JM, et al. Fast and accurate quantitative detection of Helicobater pylori and identification of clarithromycin resistance mutations in H. pylori isolates from gastric biopsy specimens by real-time PCR. J Clin Microbiol 2003; 41: 4573-4577.
7. Oleastro M, Menard A, Santos A, et al. Real-time PCR assay for rapid and accurate detection of point mutations conferring resistance to clarithromycin in Helicobater pylori. J Clin Microbiol 2003; 41: 397-402.
8. De Francesco V, Zullo A, Ierardi E, et al. Phenotypic and genotypic Helicobacter pylori clarithromycin resistance and therapeutic outcome: benefits and limits. J Antimicrob Chemother 2010; 65: 327-332.
9. Miendje Deyi VY, Bontems P, Vanderpas J, et al. Multicenter survey of routine determinations of resistance of Helicobacter pylori to antimicrobials over the last 20 years (1990 to 2009) in Belgium. J Clin Microbiol 2011; 49: 2200-2209.
10. García-Arata MI, Baquero F, de Rafael L, et al. Mutations in 23S rRNA in Helicobacter pylori conferring resistance to erythromycin do not always confer resistance to clarithromycin. Antimicrob Agents Chemother 1999; 43: 374–376.
11. Versalovic J, Osato MS, Spakovsky K, et al. Point mutations in the 23S rRNA gene of Helicobacter pylori associated with different levels of clarithromycin resistance. J Antimicrob Chemother 1997; 40: 283–286.
12. van Doorn LJ, Glupczynski Y, Kusters JG, et al. Accurate prediction of macrolide resistance in Helicobater pylori by a PCR line probe assay for detection of mutations in the 23S rRNA gene: multicenter validation study. Antimicrob Agents Chemother 2001; 45:1500-1504.
13. Stone GG, Shortridge D, Versalovic J, et al. A PCR-oligonucleotide ligation assay to determine the prevalence of 23S rRNA gene mutations in clarithromycin-resistant Helicobater pylori. Antimicrob Agents Chemother 1997; 41: 712-714.
14. De Francesco V, Margiotta M, Zullo A, et al. Clarithromycin-resistant genotypes and eradication of Helicobacter pylori. Ann Intern Med 2006; 144: 94–100.
15. Francavilla R, Lionetti E, Castellaneta S, et al. Clarithromycin-resistant genotypes and eradication of Helicobacter pylori. J Pediatr 2010; 157: 228–232.
16. De Francesco V, Margiotta M, Zullo A, et al. Primary clarithromycin resistance in Italy assessed on Helicobacter pylori DNA sequences by TaqMan real-time polymerase chain reaction. Aliment Pharmacol Ther 2006; 23: 429-435.
17. Gerard CJ, Olsson K, Ramanathan R, Reading C, Hanania EG. Improved quantitation of minimal residual disease in multiple myeloma using real-time polymerase chain reaction and plasmid-DNA complementarity determining region III standards. Cancer Res 1998; 58: 3957-3964.
18. Zullo A, De Francesco V, Hassan C, Morini S, Vaira D. The sequential therapy regimen for Helicobacter pylori eradication: a pooled-data analysis. Gut 2007; 56: 1353–1357.
19. Graham DY, Fischbach L. Helicobacter pylori treatment in the era of increasing antibiotic resistance. Gut 2010; 59: 1143–1153.
20. Vaira D, Zullo A, Vakil N, et al. Sequential therapy versus standard triple-drug therapy for Helicobacter pylori eradication: a randomized trial. Ann Intern Med 2007; 146: 556–563.

21. Neri M, Milano A, Laterza F, et al. Role of antibiotic sensitivity testing before first-line Helicobacter pylori eradication treatments. Aliment Pharmacol Ther 2003; 18: 821–827.
22. Realdi G, Dore MP, Piana A, et al. Pretreatment antibiotic resistance in Helicobacter pylori infection: results of three randomized controlled studies. Helicobacter 1999; 4: 106–112.
23. Savarino V, Zentilin P, Pivari M, et al. The impact of antibiotic resistance on the efficacy of three 7-day regimens against Helicobacter pylori. Aliment Pharmacol Ther 2000; 14: 893–900.
24. Pilotto A, Rassu M, Leandro G, Franceschi M, Di Mario F; Interdisciplinary Group for the Study of Ulcer. Prevalence of Helicobacter pylori resistance to antibiotics in Northeast Italy: a multicentre study. GISU. Interdisciplinary Group for the Study of Ulcer. Dig Liver Dis 2000; 32: 763–768.
25. De Francesco V, Margiotta M, Zullo A, et al. Prevalence of primary clarithromycin resistance in Helicobacter pylori strains over a 15 year period in Italy. J Antimicrob Chemother 2007; 59: 783–785.
26. Gisbert JP, Marcos S, Gisbert JL, Pajares JM. Helicobacter pylori eradication therapy is more effective in peptic ulcer than in non-ulcer dyspepsia. Eur J Gastroenterol Hepatol 2001; 13: 1303–1307.
27. Broutet N, Tchamgoue´ S, Pereira E, Lamouliatte H, Salamon R, Mégraud F. Risk factors for failure of Helicobacter pylori therapy – results of an individual data analysis of 2751 patients. Aliment Pharmacol Ther 2003; 17: 99–109.
28. De Francesco V, Zullo A, Hassan C, et al. The prolongation of triple therapy for Helicobacter pylori does not allow reaching therapeutic outcome of sequential scheme: a prospective, randomised study. Dig Liver Dis 2004; 36: 322–326.
29. Zullo A, Rinaldi V, Hassan C, et al. Clinical and histologic predictors of Helicobacter pylori infection recurrence. J Clin Gastroenterol 2000; 31: 38–41.
30. Miendje Deyi VY, Burette A, Bentatou Z, et al. Practical use of GenoType® HelicoDR, a molecular test for Helicobacter pylori detection and susceptibility testing. Diagn Microbiol Infect Dis 2011; 70: 557–560.