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Phenotypic and genotypic *Helicobacter pylori* clarithromycin resistance and therapeutic outcome: benefits and limits

Vincenzo De Francesco[1], Angelo Zullo[2], Enzo Ierardi[1]*, Floriana Giorgio[1], Federico Perna[3], Cesare Hassan[2], Sergio Morini[2], Carmine Panella[1] and Dino Vaira[3]

[1]Section of Gastroenterology, Department of Medical Sciences, University of Foggia, Foggia, Italy; [2]Gastroenterology and Digestive Endoscopy, 'Nuovo Regina Margherita' Hospital, Rome, Italy; [3]Department of Internal Medicine and Gastroenterology, University of Bologna, Bologna, Italy

*Corresponding author. Fax: +39-0881733848; E-mail: enzo.ierardi@fastwenet.it

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Introduction: Primary clarithromycin resistance is increasing worldwide, and it has been regarded as the main factor reducing the efficacy of *Helicobacter pylori* therapy. However, the clinical consequence of either phenotypic or genotypic resistance still remains unclear. This study aimed to evaluate: (i) the concordance between phenotypic (culture) and genotypic (real-time PCR) tests in assessing primary clarithromycin resistance; and (ii) the role of both in therapeutic outcome.

Methods: A *post hoc* subgroup study was selected from a double-blind, placebo-controlled trial, enrolling 146 patients with dyspepsia or peptic ulcers never previously treated. Real-time PCR and Etest on bacterial culture for assessing clarithromycin resistance were performed. [^{13}C]urea breath test (UBT), histology and rapid urease tests at entry and UBT after 4–8 weeks were used to assess infection and eradication. All patients received a 10 day therapy.

Results: Prevalence of clarithromycin phenotypic resistance was significantly lower as compared with genotypic resistance (18.4% versus 37.6%, $P<0.001$). A concordance between the two methods was present in 71.2% of cases. A significant difference in the eradication rate was seen between clarithromycin-susceptible and -resistant strains, when assessed with either Etest (92.4% versus 55.5%, $P<0.001$) or a PCR-based method (94.5% versus 70.9%; $P<0.001$). Of note, the eradication rate showed the lowest value (30.7%) when phenotypic bacterial resistance was genetically linked to the A2143G point mutation.

Conclusions: This study showed that: (i) there is a relevant discordance between the two methods; and (ii) phenotypic clarithromycin resistance markedly reduces *H. pylori* eradication when it is linked to a specific point mutation.

Keywords: *H. pylori*, real-time PCR, bacterial culture, Etest

Introduction

Clarithromycin is recognized as the key antibiotic for *Helicobacter pylori* treatment, having the most powerful bacteriostatic effect *in vitro* compared with the other available molecules.[1] Unfortunately, primary clarithromycin resistance is increasing worldwide, and it has been regarded as the main factor reducing the efficacy of *H. pylori* eradication therapy.[2] Clarithromycin resistance assessment is currently based on phenotypic detection performed after culture and agar dilution method or Etest.[3] However, in the past decade, different PCR-based approaches have been developed as alternative tools. These techniques allow assessment of mutations in the peptidyltransferase region encoded in domain V of the *H. pylori* 23S ribosomal RNA region conferring clarithromycin resistance.[4–7] Undeniably, both culture and PCR-based methods share advantages and limitations. Bacterial culture allows a global evaluation of *H. pylori* clarithromycin resistance, irrespective of the intrinsic mechanism involved (point mutations, RNA methylations, efflux pumps, etc.).[1,8–10] Nevertheless, *H. pylori* is a quite fastidious bacterium and culture may be difficult even in expert hands.[11] Indeed, sensitivity values of culture as low as 55%–73% have been reported in some trials.[12–15] On the other hand, PCR-based, culture-free techniques are highly accurate in finding even minimal traces of genotypic resistant strains. Moreover, PCR-based tools are accurate in detecting heteroresistant

status defined as the co-existence of strains susceptible and resistant to the same antibiotic in the same patient.[16,17] In addition, these techniques may be used even on paraffin embedded tissue.[7,18,19] Nevertheless, these approaches are unable to detect clarithromycin resistance when it is based on uncommon genetic mechanisms (deletions, RNA methylations, etc.).[1,8–10] Although several mutations have been detected, it has been found that three point mutations—namely A2143G, A2142G and A2142C—are responsible for >90% of cases of primary clarithromycin resistance in *H. pylori* strains isolated in Western countries.[20–22] Some studies have showed that these point mutations are associated with different MIC values for clarithromycin resistance assessed by culture *in vitro*,[23–25] suggesting a possible different impact on therapeutic outcome. Indeed, we previously found that the presence of the A2143G mutation—but not A2142G or A2142C—significantly lowered the *H. pylori* eradication rate.[26,27] However, the available data are still scanty.

Based on these considerations, the relative role of either phenotypic or genotypic clarithromycin resistance assessment on therapeutic outcome should be further investigated. Therefore, we designed the present study aimed at evaluating: (i) the concordance between culture and real-time PCR on primary clarithromycin resistance assessment; and (ii) the impact of both phenotypic and genotypic resistance on therapeutic outcome.

Methods

Study design

Clinical outcome

Eradication of *H. pylori* infection in patients with either clarithromycin-susceptible or resistant strains

Design

Post hoc subgroup study from a double-blind, placebo-controlled trial.[28]

Patients

One hundred and forty-six patients with dyspepsia or peptic ulcer never previously treated.

Setting

One centre (Bologna) in Italy, between September 2003 and April 2006.

Measurements

Real-time PCR and Etest on bacterial culture for assessing clarithromycin resistance, and urea breath test (UBT), histology, rapid urease tests at entry and two UBTs after 4–8 weeks to assess eradication. Paraffin-embedded gastric biopsies were retrieved and used to assess primary clarithromycin resistance using TaqMan real-time PCR.

Intervention

Either a 10 day standard triple therapy or a 10 day sequential therapy.

Ethics approval and patient consent

The initial study was approved by the Ethics Committee of the University of Bologna, Italy.[28] Patients gave written, informed consent to participate in a double-blind, therapeutic trial with multiple gastric biopsies performed at entry. The present study was performed by retrieving paraffin-embedded biopsies.

Culture and Etest

As reported in the original trial,[28] biopsies collected for bacterial culture were streaked onto Columbia agar enriched with 5% horse blood, containing vancomycin, trimethoprim, polymyxin B and nalidixic acid to inhibit the growth of microbes other than *H. pylori*. The plates were incubated in a microaerobic environment at 37°C for 7 days, and inspected daily from the third day. The isolates were identified by Gram's stain and by oxidase, catalase and urease tests. Suspensions from primary plates were prepared in sterile saline solution to have $\sim 10^8$ cells/mL, an amount sufficient to perform the Etest. A blood agar plate (Müller–Hinton with 5% horse blood without any antibiotics) was streaked in three directions with a swab dipped into each bacterial suspension to produce a lawn of growth. Etest strips (AB Biodisk, Solna, Sweden) were placed onto separate plates, which were immediately incubated in a microaerobic atmosphere at 37°C for 72 h. Isolated strains were tested for clarithromycin resistance, using as breakpoint an MIC of ≥1 mg/L.

TaqMan real-time PCR

The technique was performed in a blinded manner in a single centre (Foggia). 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 10 μm), which are largely accepted to constitute a reliable substrate for DNA analysis similar to fresh material.[20,22] The A2142C, A2142G and A2143G point mutations were analysed by a novel method (TaqMan real-time PCR) that was first used for detection of *Mycobacterium tuberculosis* and then for *H. pylori* DNA sequencing on paraffin-embedded samples, as we have recently reported.[27] Briefly, real-time PCR was performed in 96-well plates using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Monza, Italy); data collection and analyses were carried out using the specific software (same provider). The final reaction volume (25 μL) was analysed in triplicate (three samples for each patient) and all experiments were repeated twice. Primers and probes for allelic discrimination of mutant genes are given in Table 1. The result of the analysis yields three major clusters corresponding to the three genotypes: homozygous wild-type; homozygous

Table 1. Genetic sequences

23SA2142G–2142F	TCAGTGAAATTGTAGTGGAGGTGAAAA
23SA2142G–2142R	CAGTGCTAAGTTGTAGTAAAGGTCCA
VIC	AAGACGGAAAGACC for DNA wild-type
FAM	AAGACGGGAAGACC for mutated DNA
23SA2143G–2143F	TCAGTGAAATTGTAGTGGAGGTGAAAA
23SA2143G–2143R	CAGTGCTAAGTTGTAGTAAAGGTCCA
VIC	AAGACGGAAAGACC for DNA wild-type
FAM	CAAGACGGAGAGACC for mutated DNA
23SA2142C–2142F	TCAGTGAAATTGTAGTGGAGGTGAAAA
23SA2142C–2142R	CAGTGCTAAGTTGTAGTAAAGGTCCA
VIC	AAGACGGAAAGACC for DNA wild-type
FAM	AGACGGCAAGACC for mutated DNA

and heterozygous mutated type; or heteroresistant. Such a technique allows bacterial strains to be separated into three different types: susceptible, resistant and heteroresistant (i.e. mixture of resistant and susceptible strains).[16] The operator who performed all of the PCR procedures was blinded with regard to culture results. In our experience, the variability between duplicates and triplicates within the same run or different runs was usually between 0% and 2%, in agreement with previous reports.[29]

Statistical analysis

The univariate analysis was performed by using χ^2 test and Fisher's exact test, as appropriate. A model of multivariate logistic regression analysis was performed using the therapeutic outcome as the dependent variable. As possible candidates for the multivariate model, age, sex, smoking habit, alcohol intake, body mass index (BMI), endoscopic finding (peptic ulcer or non-ulcer dyspepsia), UBT δ-value and clarithromycin resistance status assessed by either culture or real-time PCR were considered. In detail, in the multivariate analysis, macrolide resistance was evaluated as the discrete variable as follows: (i) clarithromycin resistance at culture; (ii) clarithromycin resistance at PCR; and (iii) different genotypes at PCR. Variables were kept in the model only if their association with the eradication term improved the fit of the model. The odds ratio (OR) and 95% confidence interval (CI) were also calculated. Differences were considered significant at the 5% probability level. Analyses were performed using the Statsoft 7.1 program for Windows XP.

Results

Clarithromycin resistance assessment

Baseline demographic and clinical characteristics of patients are provided in Table 2. At bacterial culture with Etest, 27 *H. pylori* strains isolated were resistant to clarithromycin and 119 were susceptible, whilst the real-time PCR detected 55 clarithromycin-resistant strains and an absence of mutations in the remaining 91 strains. Therefore, the prevalence of clarithromycin phenotypic resistance was significantly lower as compared with genotypic resistance (18.4% versus 37.6%, $P<0.001$). In detail, PCR found a heteroresistant status (mixture of susceptible and resistant strains) in 42 (76.3%) out of 55 clarithromycin-resistant strains. Among these 55 resistant strains, the A2143G point mutation was present in 35 (63.6%) cases, the A2142G point mutation was present in 16 (29.1%) cases and the A2142C point mutation was present in 1 (1.8%) case, whilst two point mutations, A2143G+A2142G, were detected in the remaining 3 (5.5%) cases.

Table 2. Baseline, demographic and clinical characteristics of patients

Number of patients	146
Age (years), mean ± SD	50 ± 14.9
Sex, male/female	55/91
Smoking habit (yes/no)	37/109
PUD/NUD	11/135
Alcohol intake (yes/no)	39/107
BMI, median (range)	24.0 (16.9–34.9)
UBT δ-value, median (range)	40.3 (5.7–204.5)

PUD, peptic ulcer disease; NUD, non-ulcer dyspepsia.

Concordance between culture and real-time PCR

The segregation of resistance detected with both tools is summarized in Figure 1. In the clarithromycin-resistant group identified by culture (27 cases), PCR assessment agreed in 20 cases (11 resistant and 9 heteroresistant strains) and disagreed in the remaining 7, whilst in the susceptible group (119 cases), PCR found 84 susceptible and 35 mutated strains (2 resistant and 33 heteroresistant). Overall, a concordance between the two methods was present in 104 (71.2%) out of 146 cases. In detail, 33 (78.5%) out of 42 discordant cases were characterized by heteroresistant status at PCR and clarithromycin susceptibility at culture. By excluding all of the 42 cases in whom a heteroresistant status was found, the data of 104 patients were available. Culture identified clarithromycin-resistant strains in 18 patients and susceptible strains in 86 patients. The PCR-based assessment confirmed a mutated strain in 11 (61.1%) out of 18 resistant cases and a susceptible strain in 84 (97.7%) out of 86 susceptible cases. Therefore, the exclusion of heteroresistant status increased the concordance between the two methods up to 91.3% (95 out of 104 cases). Clarithromycin resistance assessment at culture emerged as significantly related to A2143G mutated genotype ($P<0.001$; OR 2.29, CI 2.1–8.2).

Clarithromycin resistance assessment and therapeutic outcome

H. pylori eradication was achieved in 125 (85.6%) patients. The infection was cured in 15 out of 27 patients with a clarithromycin-resistant strain and in 110 out of 119 patients harbouring a susceptible strain, as assessed by culture (55.5% versus 92.4%, $P<0.001$; OR: 0.10, 95% CI: 0.03–0.28). *H. pylori* eradication was achieved in 39 out of 55 resistant strains and in 86 out of 91 patients with susceptible strains, as assessed by PCR (70.9% versus 94.5%; $P<0.001$; OR: 0.14, 95% CI: 0.04–0.41).

As far as genotypic resistance is concerned, the data showed that *H. pylori* infection was cured less frequently in patients with a pure resistant strain as compared with those patients with heteroresistant status (6 out of 13 versus 33 out of 42 cases; 46.1% versus 78.5%, $P<0.05$) who, in turn, achieved a lower eradication rate as compared with patients infected with clarithromycin-susceptible strains (33 out of 42 cases versus 86 out of 91 cases; 78.5% versus 94.5%, $P<0.01$). According to the point mutation involved, *H. pylori* eradication was achieved in 25 (65.7%) out of 38 patients harbouring the A2143G mutated strain and 14 (82.3%) out of 17 with the remaining two mutated genotypes ($P>0.05$). Of note, the eradication rate in those patients with the A2143G mutated strains was distinctly lower as compared with patients infected with susceptible strains (65.7% versus 94.5%, $P<0.001$; OR: 8.94, 95% CI: 2.90–27.50), whilst no statistically significant difference was observed in the presence of the other two point mutations cumulatively considered (82.3% versus 94.5%, $P>0.05$; OR: 0.27, 95% CI: 0.05–1.26).

No significant difference in the eradication rate emerged according to sex, smoking habit, BMI, alcohol intake and gastroduodenal disease. The multivariate logistic regression showed that the presence of either phenotypic clarithromycin resistance ($P<0.001$; OR: 15.8, 95% CI: 4.3–24.2) or the presence of the A2143G point mutation ($P<0.05$; OR: 6.81, 95% CI: 2.2–17.8)

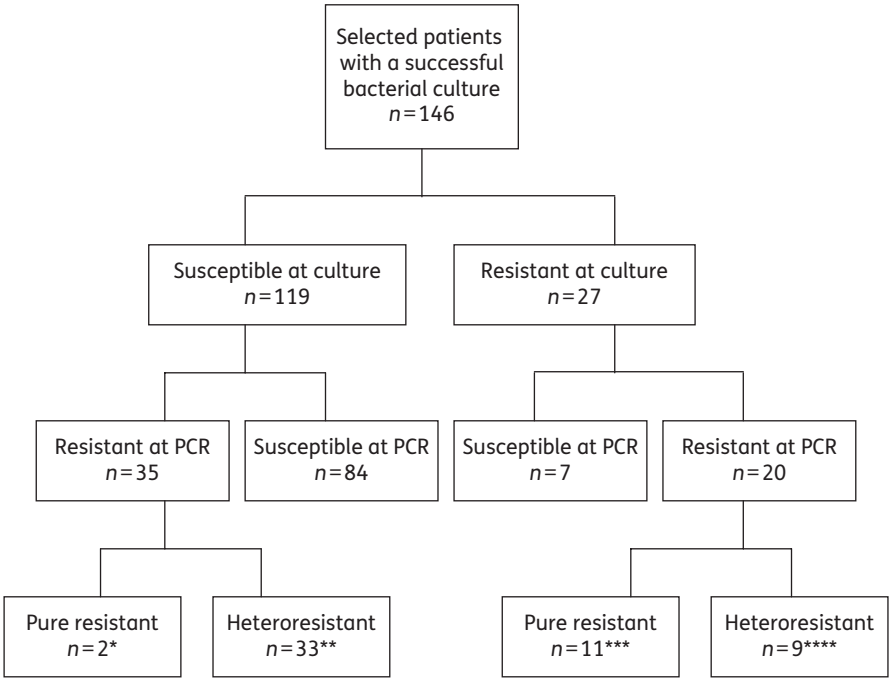

Figure 1. Segregation of primary *H. pylori* clarithromycin resistance according to culture and PCR. *2 A2143G; **22 A2143G, 10 A2142G and 1 A2143G+A2142G; ***8 A2143G, 1 A2142G, 1 A2142C, 1 A2143G+A2142G; ****3 A2143G, 5 A2142G and 1 A2143G+A2142G.

Table 3. Therapy outcome according to the concordance of phenotypic and genotypic resistance status

Clarithromycin resistance status	Cure rate (%)
Phenotypic and genotypic susceptibility	81/84 (96.4)
Phenotypic resistance and genotypic susceptibility	5/7 (71.4)
Phenotypic susceptibility and genotypic resistance (24 A2143G, 10 A2142G and 1 A2143G+A2142G)	30/35 (85.7)
Phenotypic and genotypic resistance (11 A2143G, 6 A2142G, 1 A2142C and 2 A2143G+A2142G)	9/20 (45.0)
Phenotypic and genotypic resistance with the A2143G point mutation (11 A2143G and 2 A2143G+A2142G)	4/13 (30.7)
Phenotypic and genotypic resistance without the A2143G point mutation (6 A2142G and 1 A2142C)	4/7 (57.1)

were significantly correlated with therapeutic failure. Finally, therapeutic outcome distinctly changed according to different phenotypic and genotypic clarithromycin resistance combinations. In detail, as shown in Table 3, the eradication rate dropped to only 30.7% when phenotypic bacterial resistance was genetically linked to the A2143G point mutation.

Discussion

The first relevant finding of the present study was that the genotypic clarithromycin resistance rate assessed by PCR (37.6%) was 2-fold higher compared with that of phenotypic resistance detected by bacterial culture (18.4%). Such a discrepancy most likely depends on the possible mechanisms involved in clarithromycin resistance. In fact, the PCR method is able to find bacterial strains harbouring genotypic resistance that does not emerge phenotypically.[6,7,10] On the other hand, phenotypic resistance with genotypic susceptibility could be due to either the presence of point mutations not investigated in our study[1,30] or other genetic mechanisms, such as efflux pumps or RNA methylation.[8–10] Of note, we found that the discordance between the two methods is mainly due to the detection of a heteroresistant status by PCR. Indeed, by excluding from the analysis the heteroresistant isolates, a good concordance between culture and PCR was present, confirming previous observations obtained by using a molecular immunofluorescence-based method (FISH).[7]

From a clinical point of view, it was relevant to establish whether PCR overestimated or Etest underestimated the role of clarithromycin resistance in therapeutic outcome. Our data found that genotypic resistance that failed to appear phenotypically at culture is associated with *H. pylori* eradication in >80% of patients. Therefore, the high sensitivity of the biomolecular method in assessing even minimal traces of resistant strains—as in the heteroresistant status—does not seem to meaningfully affect therapeutic outcome. On the contrary, when genotypic resistance is phenotypically expressed, the cure rate is markedly reduced to a value of 45%. On this basis, the biomolecular method could overestimate the resistance rates, strongly affecting the therapeutic options. It is noteworthy that we also observed that when phenotypic resistance is due to a specific point mutation—namely A2143G—the eradication rate further falls to a value as low as 30.7%. Such a finding clarifies the clinical role of the A2143G point mutation, which has been alternatively related to either a high or a low MIC value *in vitro*.[24,25]

Although data regarding the role of both A2142G and A2142C point mutations on the therapeutic outcome should be considered with caution due to the small sample size, our study suggests they have a marginal role in conferring real phenotypic resistance. Therefore, they probably correspond to mutational events without any clinical relevance in the management of *H. pylori*, as suggested previously.[23–27]

In our study, the phenotypic and genotypic resistance combination was present in 20 (13.7%) out of 146 cases, and this value, comparable to that observed by culture (18.4%), should probably be considered as the actual primary resistance rate affecting treatment results in our population. This value is lower than the overall prevalence of clarithromycin resistance reported by PCR in this study (37.6%) as well as in our previous study.[20]

Finally, different factors, such as smoking habit, gastroduodenal pathology, BMI, UBT δ-values and treatments, have been suggested to be involved in eradication therapy failure.[31–34] In the present study—in which the data of a double-blind trial were analysed—none of these factors was identified by the multivariate analysis as independently associated with therapeutic outcome. Indeed, only clarithromycin resistance significantly affected therapeutic success, an eradication rate as high as 96.4% being achieved in the presence of phenotypic and genotypic susceptible strains.

In conclusion, our data showed that culture-based and PCR-based methods should not be considered as alternative tools for clarithromycin resistance assessment. Moreover, we found that eradication therapy efficacy is markedly reduced when phenotypic clarithromycin resistance is sustained by the A2143G point mutation.

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Transparency declarations

None to declare.

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