Change of point mutations in *Helicobacter pylori* rRNA associated with clarithromycin resistance in Italy

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Primary clarithromycin resistance is the main factor affecting the efficacy of *Helicobacter pylori* eradication therapies (Graham & Fischbach, 2010). Genotypic and phenotypic resistance towards clarithromycin can be assessed by PCR-based and culture-dependent (Etest, agar dilution) techniques, respectively (Best et al., 2003; Chisholm & Owen, 2008). Both methods have advantages and limitations. Biomolecular tests performed using paraffin-embedded gastric biopsies are highly accurate in identifying point mutations in the *H. pylori* 23S rRNA region, which confers clarithromycin resistance (De Francesco et al., 2006; Schmitt et al., 2013).

INTRODUCTION

Primary clarithromycin resistance is considered among the most important factors affecting the efficacy of *Helicobacter pylori* eradication therapies (Graham & Fischbach, 2010). Genotypic and phenotypic resistance towards clarithromycin can be assessed by PCR-based and culture-dependent (Etest, agar dilution) techniques, respectively (Best et al., 2003; Chisholm & Owen, 2008). Both methods have advantages and limitations. Biomolecular tests performed using paraffin-embedded gastric biopsies are highly accurate in identifying point mutations in the *H. pylori* 23S rRNA region, which confers clarithromycin resistance (De Francesco et al., 2006; Schmitt et al., 2013).

Specifically, three typical point mutations (A2143G, A2142G and A2142C) have been claimed to be present in more than 90% of clarithromycin-resistant *H. pylori* isolates in Western countries (Mégraud, 2004). However, the high sensitivity of PCR-based tools in detecting even minimal traces of resistant strains may be hampered by the natural occurrence of new mutations. Conversely, culture-based methods are able to identify clarithromycin resistance irrespective of the intrinsic mechanism involved. However, *H. pylori* isolation from gastric biopsies is not always successful. Sensitivity is no more than 90% and has been reported to be 55–73% in some trials, where optimal conditions are usually encountered (Zullo et al., 2003).
On this basis, variable degrees of concordance between genotypic and phenotypic identification of clarithromycin resistance in \( H. \) pylori strains have been reported (Burucoa \textit{et al.}, 2008; De Francesco \textit{et al.}, 2010; Morris \textit{et al.}, 2005) and the choice of the best method for resistance assessment remains controversial (Malferttheiner \textit{et al.}, 2012). In addition, MIC breakpoints associated with different point mutations remain unclear (Cambau \textit{et al.}, 2009; De Francesco \textit{et al.}, 2009a; Mégraud & Lehours, 2007; Rimbara \textit{et al.}, 2008).

This study aimed: (i) to assess concordance between phenotypic and genotypic clarithromycin resistance caused by the presence of the three typical point mutations; (ii) to search for three other point mutations that have been described in Europe (A2115G, G2141A and A2144T) (Agudo \textit{et al.}, 2010; Garrido & Toledo, 2007; Toracchio \textit{et al.}, 2004) in discordant cases; and (iii) to compare the MICs associated with each point mutation.

**METHODS**

\textbf{Patients.} All patients who underwent an upper endoscopy in a single centre (University of Bologna) for dyspeptic symptoms between January 2011 and December 2012 were considered for entry into the study. Only those patients who had never been previously treated for \( H. \) pylori infection and in whom a successful bacterial culture with antibiotic susceptibility testing was available were enrolled. For the purpose of the study, both clarithromycin-susceptible and -resistant strains were selected. In order to homogenize our study sample, only \( H. \) pylori strains with exclusive clarithromycin resistance were included in the resistant group; strains with simultaneous resistance to other antibiotics were excluded, so that potential interference by other antibiotic resistances could be ruled out. Similarly, in the clarithromycin-susceptible group, only \( H. \) pylori strains without any resistance towards the tested antibiotics (clarithromycin, levofloxacin, metronidazole and amoxicillin) were included.

\textbf{Culture and Etest.} Biopsies collected for bacterial culture were streaked onto Columbia agar enriched with 5 % horse blood and containing vancomycin, trimethoprim, polymyxin B and nalidixic acid to inhibit the growth of microbes other than \( H. \) pylori. The plates were incubated in a micro-aerobic environment at 37 °C for 7 days, and inspected daily from day 3. The isolates were identified by Gram stain and by oxidase, catalase and urease tests. Suspensions from primary plates were prepared in sterile saline solution to approximately 10^8 cells ml^-1, an appropriate amount to perform the Etest. A blood agar plate (Mueller–Hinton (Biomerieux Italia SpA, Florence, Italy) with 5 % horse blood without any antibiotics) was used for 72 h. Antimicrobial resistance was assessed by using the break-point MICs for clarithromycin (>0.5 mg l^-1), amoxicillin (>0.12 mg l^-1), levofloxacin (>1 mg l^-1) and metronidazole (>8 mg l^-1), as suggested in the current European Committee on Antimicrobial Susceptibility Testing recommendations (EUCAST, 2013).

\textbf{TaqMan real-time PCR.} The genotypic procedure was performed in a single centre (University of Bari) by an operator who was blind to the phenotypic results. DNA was extracted from the retrieved gastric biopsy specimens using a NucleoSpin Tissue kit (Macherey-Nagel) and applied to paraffin-embedded sections (at least five sections of 10 μm), as reported elsewhere (De Francesco \textit{et al.}, 2006, 2007). TaqMan real-time PCR was used to search for the three most prevalent genotypic mutations (A2142C, A2142G and A2143G). In those cases with discordant results between phenotypic and genotypic findings (i.e. phenotypic resistance without any of the three tested point mutations), further searches were conducted for other, less frequent point mutations including A2144T, A2115G and G2141A, which have previously been detected in \( H. \) pylori strains isolated in Italy, the UK and Spain (Garrido & Toledo, 2007; Toracchio \textit{et al.}, 2004). Primers and probes for the allelic discrimination of mutant genes are listed in Table 1.

**Statistical analysis.** Univariate analysis was performed using a \( \chi^2 \) test and Fisher’s exact test, as appropriate. Continuous variables were compared using a Mann–Whitney U test, and log_{10} of MIC values were used for comparison. Odds ratios and their 95 % confidence intervals were also calculated. Differences were considered significant at the 5 % probability level. The agreement grade between phenotypic and genotypic resistance was evaluated using the Cohen \( \kappa \) coefficient. The agreement between genotypic and phenotypic resistance and concordance was classified as follows: <0.20, slight; 0.21–0.40, fair; 0.41–0.60, moderate; 0.61–0.79, substantial; and ≥0.80, excellent, as reported elsewhere (Landis & Koch, 1977). Analyses were performed by using the StatSoft 7.1 program for Windows xp.

**RESULTS**

Biopsies from 82 patients infected with \( H. \) pylori were analysed, including 42 strains that were clarithromycin resistant and 40 that were clarithromycin susceptible on bacterial culture (Table 2). On genotypic analysis, at least one of the three most prevalent point mutations (A2142C, A2142G and A2143G) was detected in only 23 cases (54.8 %) out of the 42 phenotypically resistant strains: 12 cases with A2143G mutations, seven with A2142G mutations and four with an A2143G plus A2142G double point mutation. In the phenotypically susceptible group, real-time PCR identified a point mutation in eight cases (20 %), with six A2142G and two A2143G mutations. The A2142C mutation was not observed in any case.

Among the 19 cases with phenotypic clarithromycin resistance but in none of the three most prevalent point mutations, real-time PCR detected at least one of the other three tested novel point mutations in a further 14 cases (73.6 %), with three A2115G mutations, six G2141A mutations, four A2144T mutations and one A2115G plus A2144T double mutation. In the phenotypically susceptible group, real-time PCR identified the A2115G point mutation in a further three cases (7.5 %). Therefore, searching for other potential mutations beyond the three most prevalent significantly increased the accuracy of PCR in detecting clarithromycin resistance in \( H. \) pylori strains from 54.7 % to 88 % (\( P<0.001; \) odds ratio 6.1, 95 % confidence interval 2–18.6). In contrast, the false-positive rate (cases with a point mutation detected on PCR but clarithromycin susceptible on culture) only slightly increased from 20 % to 27.5 % (\( P=\)not significant).

When considering only the three typical point mutations, the \( \kappa \) coefficient of concordance between PCR and culture
in assessing clarithromycin resistance was 0.67, which increased to 0.81 when the three new mutations were added. Therefore, searching for all six point mutations resulted in excellent agreement between the two tools.

No significant differences in MIC values were observed between the A2143G and A2142G mutant strains (median log10 1.0 vs 2.0, P=0.22) or between the 'typical' (cumulative) and 'novel' (cumulative) mutant strains (median log10 1.46 vs 1.47, P=0.9). The distribution of median MIC values according to the different point mutations in phenotypically resistant and susceptible strains is shown in Table 3.

**DISCUSSION**

Clarithromycin resistance plays a major role in *H. pylori* management because of its impact on therapeutic outcomes (Graham & Fischbach, 2010). Culture-dependent tools and various PCR-based methods are currently used to assess clarithromycin resistance, and each has advantages and limitations (De Francesco et al., 2010; Zullo et al., 2003). In essence, the high sensitivity of PCR-based techniques in detecting even minimal traces of mutated genotypes is balanced by the ability of culture to detect phenotypic resistance irrespective of the genetic mechanism involved. Indeed, the occurrence of novel point mutations in the V domain of bacterial rRNA can reduce the sensitivity of PCR-based methods.

The first relevant finding of this study is that the prevalence of the three most frequently reported point mutations (A2142C, A2142G and A2143G), claimed to be detectable in more than 90% of clarithromycin-resistant *H. pylori* strains (De Francesco et al., 2007; Megraud, 2004; Wuepkenhorst et al., 2009), was reduced to only 54.8%, with the A2142C mutation not being detected at all. Of note, we previously found that these three point mutations were

### Table 1. Genetic sequences

<table>
<thead>
<tr>
<th>Probes</th>
<th>Primers</th>
</tr>
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<tbody>
<tr>
<td>23SA2142G-2142F</td>
<td>5'-TCAGTGAAATTGTAGGGAGGTGAAAA-3'</td>
</tr>
<tr>
<td>23SA2142G-2142R</td>
<td>5'-CAGTGCTAAGTTGTAGTAAAGGTCCA-3'</td>
</tr>
<tr>
<td>VIC</td>
<td>5'-AAGACGGAAGACCC-3' for wild-type DNA</td>
</tr>
<tr>
<td>FAM</td>
<td>5'-GCTGTCTCAACCAAGAGAACGT-3'</td>
</tr>
<tr>
<td>23SA2143G-2143F</td>
<td>5'-TCAGTGAAATTGTAGGGAGGTGAAAA-3'</td>
</tr>
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<td>VIC</td>
<td>5'-AAGACGGAAGACCC-3' for wild-type DNA</td>
</tr>
<tr>
<td>FAM</td>
<td>5'-AGACGGAAGACCC-3' for mutated DNA</td>
</tr>
<tr>
<td>23SA2142C-2142F</td>
<td>5'-TCAGTGAAATTGTAGGGAGGTGAAAA-3'</td>
</tr>
<tr>
<td>23SA2142C-2142R</td>
<td>5'-CAGTGCTAAGTTGTAGTAAAGGTCCA-3'</td>
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<td>VIC</td>
<td>5'-AAGACGGAAGACCC-3' for wild-type DNA</td>
</tr>
<tr>
<td>FAM</td>
<td>5'-AGACGGAAGACCC-3' for mutated DNA</td>
</tr>
<tr>
<td>23SG2115G-2115F</td>
<td>5'-AGGTGAAATTCC-3' for wild-type DNA</td>
</tr>
<tr>
<td>23SG2115G-2115R</td>
<td>5'-AGGTGAAATTCC-3' for mutated DNA</td>
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<tr>
<td>VIC</td>
<td>5'-AGGTGAAATTCC-3' for wild-type DNA</td>
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<tr>
<td>FAM</td>
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<td>23SA2144T-2144F</td>
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<tr>
<td>23SA2144T-2144R</td>
<td>5'-CAGTGCTAAGTTGTAGTAAAGGTCCA-3'</td>
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<td>23SG2141A-2144F</td>
<td>5'-AGTGCTAAGTTGTAGTAAAGGTCCA-3'</td>
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<tr>
<td>23SG2141A-2144R</td>
<td>5'-CAGTGCTAAGTTGTAGTAAAGGTCCA-3'</td>
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<td>VIC</td>
<td>5'-AAGACGGAAGACCC-3' for wild-type DNA</td>
</tr>
<tr>
<td>FAM</td>
<td>5'-AAGACGGAAGACCC-3' for mutated DNA</td>
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</tbody>
</table>

### Table 2. Distribution of point mutations in phenotypically resistant and susceptible strains

The A2142C point mutation was not observed in any case.
relevant for clinical practice. Indeed, an 
H. pylori instead of three point mutations, such a finding could be
Although the rate of false-positive PCR results increased
the actual prevalence in Italy (De Francesco
performed an accurate assessment of clarithromycin resistance
least six point mutations should be searched for in order to
substantial to excellent. These observations suggest that at
and phenotypic clarithromycin resistance increased from
of three point mutations, concordance between genotypic
Consequently, the search for all six point mutations –
shift towards the ‘novel’ mutations.
Triple mutation: A2143G plus A2142G plus A2144T.
A2144T; one A2142G plus A2115G; one A2143G plus A2115G.
†Triple mutation: A2143G plus A2142G plus A2144T.

<table>
<thead>
<tr>
<th>Point mutation (n)</th>
<th>MIC value (mg l⁻¹), Median (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A2143G (11)</td>
<td>12 (3–256)</td>
</tr>
<tr>
<td>A2142G (6)</td>
<td>152 (3–256)</td>
</tr>
<tr>
<td>A2115G (1)</td>
<td>4</td>
</tr>
<tr>
<td>A2144T (3)</td>
<td>134 (8–256)</td>
</tr>
<tr>
<td>G2141A (6)</td>
<td>28 (1–256)</td>
</tr>
<tr>
<td>Double mutations (6)†</td>
<td>4 (0.5–256)</td>
</tr>
<tr>
<td>Triple mutations (1)†</td>
<td>256</td>
</tr>
</tbody>
</table>

*Double mutations: three A2143G plus A2142G; one A2115G plus A2144T; one A2142G plus A2115G; one A2143G plus A2115G.
†Triple mutation: A2143G plus A2142G plus A2144T.

present in 74 % of phenotypically resistant strains isolated from patients in the same geographical area between 2003 and 2006 (De Francesco et al., 2010). In addition, we observed that novel point mutations are emerging in 
H. pylori isolates. In the current study, three other point mutations that have already been described in Western countries (i.e. A2144T, A2115G and G2141A) were present in a further 14 out of 19 phenotypically clarithromycin-resistant 
H. pylori strains. Therefore, these preliminary observations suggest a relative reduction in the prevalence of the typical point mutations in Italy, with a progressive shift towards the ‘novel’ mutations.

Consequently, the search for all six point mutations – instead of the three ‘classical’ mutations – resulted in a 6.1-fold increased probability of detecting genotypic resistance in phenotypically resistant strains. Similarly, with six instead of three point mutations, concordance between genotypic and phenotypic clarithromycin resistance increased from substantial to excellent. These observations suggest that at least six point mutations should be searched for in order to perform an accurate assessment of clarithromycin resistance in 
H. pylori using a PCR-based method. Based on this observation, the overall rate of primary clarithromycin resistance (10 %) that we recently computed exclusively using a PCR-based method for the three typical point mutations, most likely represents an underestimation of the actual prevalence in Italy (De Francesco et al., 2011a). Although the rate of false-negative PCR results increased only slightly from 20 % to 27.5 % when searching for six instead of three point mutations, such a finding could be relevant for clinical practice. Indeed, an 
H. pylori strain could be fallaciously classified as clarithromycin resistant, affecting the therapeutic options. It could be useful to compare clarithromycin resistance as assessed by PCR on DNA extracted from biopsies to that from colonies showing resistance to the agent in a future study.

Another relevant finding of the present study is that no statistically significant differences emerged among the MIC values of clarithromycin resistance associated with different point mutations. Specifically, no differences emerged between the ‘classical’ and ‘novel’ mutations, suggesting a similar potential impact on 
H. pylori management. In previous studies, high MIC values have been alternatively associated with either the A2143G or the A2142G point mutation (De Francesco et al., 2009b, 2011b; Mégraud, 2004; Owen, 2002). Of note, our data failed to find a single mutation associated with consistently high MIC values. This suggests that phenotypic resistance may depend on multiple and synergic genetic mechanisms (Agudo et al., 2010; De Francesco et al., 2011b; Liu et al., 2008), rather than being an exclusive consequence of a specific point mutation.

In conclusion, new point mutations responsible for 
H. pylori clarithromycin resistance are emerging in Italy. Similar MIC values are associated with different mutant genotypes. PCR-based methods should search for at least six point mutations to achieve good accuracy in detecting clarithromycin resistance. Therefore, phenotypic resistance assessed by culture, irrespective of the genetic mechanism involved, remains the gold standard for clinical practice.

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