Clarithromycin resistance of *Helicobacter pylori* has a major impact on the efficacy of the omeprazole-amoxicillin-clarithromycin therapy

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Summary

Clarithromycin resistance of *Helicobacter pylori* is relatively frequent in France and is assumed to be the main cause of failure of the proton pump inhibitor-amoxicillin-clarithromycin (PPI-AC) therapy, which is the first-line regimen in our country. We determined the respective effects of clarithromycin primary and secondary resistances on efficacy of the PPI-AC regimen and examined whether failures were associated with persistence of the same strain or with emergence of a new one. Hundred and twenty three *H. pylori*-infected patients were treated for seven days with omeprazole 20 mg b.d., amoxicillin 1g b.d., and clarithromycin 500 mg b.d. Eradication was assessed by breath test in 102 patients. MICs of clarithromycin were determined by E-test. Strain genotyping was performed by random amplified polymorphic DNA. The pre-treatment and post-treatment prevalences of clarithromycin resistance were 18.7% (23/123) and 69.2% (9/13), respectively. The rates of eradication were 67.6% (69/102), 78.8% (67/85), and 11.8% (2/17) for all, susceptible and resistant strains, respectively. The post-treatment isolate was available for six patients with a susceptible pre-treatment isolate and a persistent infection; resistance emerged in two patients and was associated with persistence of the pre-treatment strain in one and with selection of a new strain in the other. In conclusion, in our hospital, failures of the PPI-AC therapy are related to both clarithromycin primary and secondary resistances but emergence of secondary resistance does not explain all failures in the initial clarithromycin-susceptible group. In that group a new strain can emerge after failure. © 2001 Éditions scientifiques et médicales Elsevier SAS

clarithromycin / *Helicobacter pylori* / prevalence / resistance / treatment

Résumé – La résistance à la clarithromycine de *Helicobacter pylori* et ses conséquences sur l’efficacité du traitement par oméprazole-amoxicilline-clarithromycine.

La résistance à la clarithromycine de Helicobacter pylori est relativement fréquente en France et est considérée comme la principale cause d’échec du traitement par inhibiteur de la pompe à protons, amoxicilline et clarithromycine (IPP-AC), utilisé en première ligne dans notre pays. Nous avons déterminé les conséquences respectives des résistances primaire et secondaire à la clarithromycine sur l’efficacité du traitement IPP-AC et avons cherché à savoir si les échecs d’éradication s’associaient à la persistance de la souche initiale ou à l’émergence d’une nouvelle souche. Cent vingt-trois patients infectés par *H. pylori* ont été traités pendant sept jours par oméprazole 20 mg 2 × j, amoxicilline 1 g 2 × j et clarithromycine 500 mg 2 × j. Le succès du traitement d’éradication a été apprécié par un test respiratoire chez 102 patients. Les CMI de la clarithromycine ont été déterminées par E-test. Le génotypage des souches a été effectué par la technique de RAPD. Les prévalences de résistance à la clarithromycine avant et après traitement étaient respectivement de 18,7% (23/123) et 69,2% (9/13). Les taux d’éradication observés pour toutes les souches, les souches sensibles et les souches résistantes étaient respectivement de 67,6% (69/102), 78,8% (67/85) et 11,8% (2/17). L’isolat

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The one-week triple regimen amoxicillin 1 g b.d.-clarithromycin 500 mg b.d.-proton pump inhibitor (PPI) at a double dose is widely prescribed as eradication therapy for Helicobacter pylori. In the international multicentric MACH1 [1] and MACH2 [2] studies intention-to-treat eradication rates higher than 90% were found with this regimen. However, studies performed in some countries including the United States and France led to less impressive results, with success rates varying from 66 to 86% [3–8] and from 56 to 84% [9–11] in U.S. and French studies, respectively.

It is accepted that resistance to clarithromycin is the major factor implicated in these failures. The prevalence of primary resistance to clarithromycin appears to be low, less than 10%, in many countries [12]. However, it is estimated to be higher than 10% in Belgium [12] and France [13]. Prevalences of clarithromycin resistance higher than 10% have also been reported in studies from Peru [14], Poland [15], the United States [16, 17], Hong Kong [18] and Japan [19]. The data concerning the influence of primary clarithromycin resistance on eradication with the amoxicillin-clarithromycin-PPI suggest that it may cause a significant decrease in the eradication rate [5, 10, 20–22]. However, small numbers of resistant isolates were studied in most reports. The data concerning the prevalence of clarithromycin resistance after failure of this triple therapy are conflicting, prevalences varying from 0% to 69% [2, 5, 23] and again, in most reports only a limited number of isolates were examined. Finally, the respective contributions of primary and secondary resistances in treatment failures have not been determined. We therefore aimed to better understand relationships between clarithromycin primary and acquired resistances and failure of the amoxicillin-clarithromycin-PPI triple therapy. We also determined whether eradication failure was associated with persistence of the same strain or with emergence of a new one.

MATERIALS AND METHODS

Study design

H. pylori infection was diagnosed from biopsy samples routinely taken by gastroscopy from patients referred to our center for ulcer-like dyspepsia. Three endoscopic biopsies taken from the antrum (three cm from the pylorus) and three from the fundus were sent in formalin for histology. In addition, one biopsy from the antrum and one from the fundus were immediately frozen and kept at −80°C for culture. Infection by H. pylori was defined as positivity of histology and culture. All patients received one-week triple therapy with omeprazole 20 mg b.d., amoxicillin 1 g b.d., and clarithromycin 500 mg b.d. (OAC). H. pylori eradication was assessed by 13C-urea breath test four to six weeks after the end of treatment. In case of failure, patients underwent a second endoscopy and one biopsy from the antrum and one from the fundus were taken for culture.

Histological analysis with grading of H. pylori density and gastritis activity

Formalin-fixed mucosal samples were stained with cresyl fast violet. The bacterial density was estimated in the mucosa layer, the epithelium, and the glands. A score ranging from 1 to 3 was established as follows: 1, less than 10 bacteria per gland; 2, 10 to 20 bacteria in at least one gland; and 3, more than 20 bacteria in at least one gland or over the superficial epithelium. Gastritis activity defined by neutrophil infiltration was graded in antrum and fundus as 1: absent, 2: mild, 3: moderate, and 4: severe according to the updated Sydney system [24]. An overall score was established by the sum of these grades in antrum and fundus.

Culture and antibiotic susceptibility testing

Two biopsies from each patient (one from the antrum and one from the fundus) were immersed together into one mL of sterile water and homogeneized for ten seconds with the Ultra Turrax T25™ apparatus (Janke and Kunkel, Staufen, Germany). Biopsy homogenates were cultured on a commercialized serum-supplemented selective medium, Pylori agar (bioMérieux, Marcy l’Étoile, France) under microaerophilic conditions at 37°C for a maximum of six days. H. pylori isolates were defined as gram-negative spiral-shaped bacilli that were oxidase positive and rapidly (less than one hour) urease positive. Minimal inhibitory concentrations (MICs) of clarithromycin and metronidazole were determined by the E-test method (AB Biodisk, Solna, Sweden) according to the instructions of the manufacturer, using Mueller-Hinton agar (Oxoid, Lyon, France) supplemented with 10% horse

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### Table I. Prevalences of pre-treatment and post-treatment resistance of *H. pylori* to clarithromycin, metronidazole, and both antibiotics.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Pre-treatment</th>
<th>Post-treatment</th>
<th>Post-treatment (plus 47 additional patients)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>clarithromycin</td>
<td>18.7% (23/123)</td>
<td>69.2% (9/13)</td>
<td>66.7% (40/60)</td>
</tr>
<tr>
<td>metronidazole</td>
<td>43.7% (28/64)</td>
<td>45.4% (5/11)</td>
<td>53.4% (31/58)</td>
</tr>
<tr>
<td>clarithromycin and metronidazole</td>
<td>14.1% (9/64)</td>
<td>45.4% (5/11)</td>
<td>43.1% (25/58)</td>
</tr>
</tbody>
</table>

*The pre-treatment isolate was not available for these 47 additional patients.*

Blood (bioMérieux) and a cell suspension calibrated at 3 McFarland units. Plates were read after three days of incubation at 37°C as described above. Strains were considered resistant to clarithromycin if the MIC was > 0.5 µg/mL [25] and resistant to metronidazole if the MIC was > 4 µg/mL [26].

**Genotyping of pre- and post-treatment paired isolates obtained from the same patient**

Target chromosomal DNA was extracted from *H. pylori* strains using the QIAamp Tissue Kit (Qiagen, Courtaboeuf, France). Random Amplified Polymorphic DNA (RAPD), with a single 11-bp oligonucleotide (AGT-TCAGCCAC), was used to determine the genetic relatedness of paired isolates [27]. PCR was performed in a 100-µl volume containing 10 mM Tris-HCl (pH 8.3) (Boehringer Mannheim, Meylan, France), 1.5 mM MgCl₂, 50 mM KCl, 0.25 mM of each deoxynucleotide (Pharmacia Biotech, Uppsala, Sweden), 1 µl of DNA sample, 2.5 units of Taq DNA polymerase (Boehringer Mannheim), and 3 µM of the oligonucleotide primer. Amplification was carried out using a GeneAmp PCR System 9600 thermal cycler (Perkin-Elmer Biosystems, Courtaboeuf, France) programmed for 45 consecutive cycles consisting of a denaturation step of 94°C for 1 min, a primer annealing step of 36°C for 1 min, and an extension step of 72°C for 2 min. Amplification products were analyzed by electrophoresis on a 2% agarose gel. Isolates were considered to be genetically related if their profiles did not differ by more than one band.

**Statistical analysis**

Comparisons between eradication success and clarithromycin susceptibility/resistance, bacterial density or neutrophil infiltration grades were based on univariate analysis using Pearson’s chi-square test or Fisher’s exact test.

**RESULTS**

**Prevalence of resistance before treatment**

Between January 1996 and September 1999, 123 subjects, mean age 49.3 years (range 18–77 years), 85 (69.1%) of whom were men, underwent an endoscopy and were *H. pylori*-positive by culture and histology. The patients had either gastroduodenal ulcer (77, 62.6%), MALT (8, 6.5%) or non-MALT (2, 1.6%) lymphoma of the stomach, or non ulcer dyspepsia (36, 29.3%). The prevalence of primary resistance to clarithromycin found in our center was 18.7% (23/123) (table I). There was no statistical relationship between clarithromycin susceptibility patterns and endoscopic diagnoses. The susceptibility to metronidazole was also determined for 64 of the 123 study strains and the prevalence of resistance was 43.7% (28/64) (table I).

**Rates of eradication**

The 123 *H. pylori*-infected patients included in the study received one-week triple therapy with amoxicillin 1 g b.d., clarithromycin 500 mg b.d., and omeprazole 20 mg b.d. *H. pylori* eradication was assessed by breath-test for 102 of them (82.9%) with an eradication rate of 67.6% (69/102) (figure 1). The eradication rate was found to be very low, 11.8%, (2/17) when the pre-treatment strain was clarithromycin-resistant, as compared to 78.8% (67/85) (figure 1). The eradication rates were 60.0% (6/10), 69.6% (16/23), 68.1% (47/69) for patients with bacterial densities graded 1, 2, and 3, respectively. They were 73.3% (11/15), 63.9% (23/36), 69.2% (27/39), and 66.7% (8/12) for patients with gastritis activities graded 0, 1, 2, and 3, respectively. Bacterial density and gastritis activity were not statistically linked to the eradication rate.

**Prevalence of resistance after treatment and genotyping of the strains**

Six of the 18 patients with a clarithromycin-susceptible *H. pylori* pre-treatment isolate and a persistent infection after treatment had post-treatment isolates available for susceptibility testing: only two developed clarithromycin resistance (figure 1). Seven of the 15 patients with a clarithromycin-resistant *H. pylori* pre-treatment isolate and a persistent infection after treatment had post-treatment isolates available for susceptibility testing and all these remained clarithromycin-resistant (figure 1).
In order to determine whether the two members of each of the above-described 13 pairs of pre- and post-treatment isolates corresponded to the same strain, they were subjected to genotypic typing by RAPD. For the seven pairs with a clarithromycin-resistant pre-treatment isolate, the RAPD patterns of the two members of each pair were identical (data not shown). This showed that in case of primary resistance to clarithromycin, eradication failure was associated with persistence of the resistant strain. In contrast, when the pre-treatment isolate was clarithromycin-susceptible, in two out of five cases a new strain emerged after treatment (data not shown), one of these two strains being clarithromycin-susceptible and the other -resistant (the sixth pair could not be analyzed). The same strain persisted after treatment in the three remaining cases and clarithromycin resistance occurred in one of them.

If these post-treatment isolates were combined, 9 out of 13 (69.2%) were clarithromycin-resistant (table I, figure 1). The susceptibility to metronidazole was determined for 11 of these 13 strains: five were resistant (45.4%) and all of them were also resistant to clarithromycin (table I). The number of post-treatment isolates available being low, we tested the susceptibility to clarithromycin and metronidazole of 47 other post-treatment strains from patients of the same period [mean age 51.0 years, range 24–83 years, 32 men (68.1%)] not included in the study, but that had received the same treatment. Upon addition of these 47 strains, the prevalence of post-treatment clarithromycin resistance was 66.7% (40/60) whereas that of metronidazole resistance was 53.4% (31/58). Furthermore, 43.1% (25/58) of the strains were resistant to both antibiotics (table I).

**DISCUSSION**

The prevalence of primary resistance to clarithromycin found in our center, 18.7%, is relatively high compared to that reported in a recent nationwide national survey: 12.9% [13]. This suggests that the prevalence of primary resistance to clarithromycin may vary notably from one center to another and thus that each center should determine its own prevalence of resistance. The relatively high prevalence of clarithromycin resistance in *H. pylori* observed in France and Belgium is probably due the high consumption of macrolides in both countries [12]. 17.1% (21/123) of the patients enrolled were not subjected to breath testing, this relatively high rate of patients lost for follow-up may be explained by the fact that this study was realized during usual routine practice.

The eradication rate of the one-week OAC triple therapy was 67.6%. This rate is similar to those found recently in other French studies (range 56–84%) [9 – 11]. Interestingly, we found that primary resistance to clarithromycin led to treatment failure in almost all cases (15 out of 17 cases, eradication rate: 11.8%). Five other studies also found low eradication rates with the amoxicillin-clarithromycin-PPI regimen for a duration of seven to ten days in case of pre-treatment resistance to clarithromycin. However, most of these studies were performed with only a small number of strains. The rates observed were as follows: 0% (0/5) [10], 20% (2/10) [20], 29% (4/14) [5],
50% (4/8) [21], and 50% (3/6) [22]. The only exception was the MACH2 study: the two clarithromycin-resistant strains included were eradicated [2].

The eradication rate was not statistically linked with bacterial density nor with gastritis activity. Our findings are in keeping with those of Abdul Aal et al. who did not find a link between density estimated by histology and eradication rate [28]. In another work, *H. pylori* density has been found to be a predicting factor for eradication [29], but density was indirectly assessed by urea breath test stating that urease activity was related to density. However, this relation has not been proved and it has been shown that urease activity may substantially variate from one strain to another [30].

Thus, clarithromycin primary resistance was largely implicated in treatment failures, but in our study it explained only approximately half (15/32) of them. The 18 other cases concerned clarithromycin-susceptible pre-treatment isolates and we found that the rate of emergence of resistance under treatment was low: only two of six patients with post-treatment susceptibility testing of isolates available developed clarithromycin resistance after therapy. These results are consistent with other reports.

In one study by Laine et al. [5], of the ten patients with pre-treatment susceptible isolates who had persistent infection after a ten-day amoxicillin-clarithromycin-omeprazole therapy, only three developed clarithromycin resistance. In addition, Yousfi et al. [8], Wurzer et al. [22], and Lind et al. [2] reported that zero of six, two of six, and zero of three patients with pre-treatment clarithromycin-susceptible isolates who failed OAC therapy developed clarithromycin resistance, respectively. If all these cases as well as ours are taken together, clarithromycin resistance emerged in only seven out of 31 patients (22.6%). This indicates that eradication failures are rarely associated with emergence of resistance under treatment. We found that the prevalence of resistance after treatment was high (68.3%), illustrating the fact that in countries like France where the prevalence of primary resistance to clarithromycin is relatively high, post-treatment resistance is frequent but is mostly due to persistence of a pre-treatment resistant strain rather than to emergence of resistance under treatment.

Another question was: in case of failure, does the same strain persist after treatment and, if a new strain appears, is this phenomenon linked to clarithromycin resistance? The genotyping experiments performed suggest that when the strain is susceptible before treatment, eradication failures are associated either with persistence of the strain or with emergence of a new strain. Reinfections being rare, it is likely that this emergence is explained by the coexistence of two strains (one predominating) before treatment and by eradication of the predominating one under treatment. As stated previously, clarithromycin resistance rarely occurs under treatment but our data suggest that it can be associated with both phenomena: persistence of the strain or occurrence of a new strain. Results of previous studies have suggested that failures of clarithromycin monotherapy or clarithromycin-PPI dual therapies were mostly associated with persistence of the same strain which had usually become clarithromycin-resistant [31–34]. However, in another report five of ten patients on clarithromycin-omeprazole dual therapy were infected by a new strain after failure, and two of the strains remained clarithromycin-susceptible [35]. Our results are quite similar and confirm that clarithromycin-based therapies, and particularly triple therapies, can select for the emergence of new strains that furthermore can remain clarithromycin-susceptible. It may be that these strains are nonetheless less susceptible to the bactericidal activities of clarithromycin and/or amoxicillin, but this remains to be determined.

Metronidazole represents an alternative in case of clarithromycin resistance. However, in our work an alarming 14.1% of pre-treatment isolates and an even more alarming 43.1% of post-treatment isolates were coresistant to clarithromycin and metronidazole. Thus, in our experience the choice of the second-line regiment is frequently difficult to make. Indeed, two recent meta-analyses have clearly established that metronidazole resistance has a notable impact on the efficacy of amoxicillin-metronidazole-PPI regimen [36, 37]. Studies are needed to determine whether higher doses of metronidazole or a longer duration of treatment are able to diminish this impact.

**CONCLUSION**

This work confirms the major negative impact of primary resistance to clarithromycin on the eradication rate of the OAC therapy and suggests that acquisition of resistance under treatment is a relatively rare event with that regimen. In addition, strain genotyping experiments indicate that in case of pretreatment resistance the same strain usually persists after failure. In contrast, in case of pretreatment susceptibility the initial strain can either persist or be replaced by a new one and both phenomena are compatible with either susceptibility or resistance to clarithromycin.

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