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Brief reports

Clarithromycin resistance stability in *Helicobacter pylori*: influence of the MIC and type of mutation in the 23S rRNA

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Thirty clarithromycin-resistant *Helicobacter pylori* strains (MIC range 8–64 mg/L) were subcultured in a drug-free medium and the MIC was determined every five passages to detect *in vitro* stability of resistance. Three out of the 30 (10%) lost their resistance after 10, 13 or 18 subcultures (MIC decrease from 8 to 0.008, from 16 to 0.064 and from 32 to 0.016 mg/L). The effect of four macrolides at subinhibitory concentrations on the development of resistance was studied in *H. pylori* NCTC 11638 and TIGR 26695. A change in the MIC was observed only when NCTC11638 was exposed to 0.5 × MIC of erythromycin for 20 days.

Introduction

Helicobacter pylori is a Gram-negative microaerophilic rod found in the human gastric mucosa and is associated with a variety of digestive diseases, such as peptic ulcer, gastritis and MALT lymphoma, and is considered a risk factor in the development of gastric cancer.[1] Amoxycillin, tetracycline, metronidazole and macrolides (mainly clarithromycin) are the most frequently used antimicrobials, combined with proton pump inhibitors or bismuth salts, for the treatment of *H. pylori* infections.[2] However, side effects, poor compliance and resistance to the antibiotics used are common causes of treatment failure.[3,4]

The prevalence of clarithromycin resistance varies with geographical location but it is generally less than 10%. In Spain, 3.5% of *H. pylori* are clarithromycin resistant, whilst in other European countries the percentage has ranged from 0 to 10%.[3,4] The mechanism of resistance to clarithromycin described in *H. pylori* is a transition mutation in the peptidyl transferase region of the 23S rRNA at *Escherichia coli*-equivalent base A2058 or A2059.[5] Point mutations in which an adenine residue was replaced by a guanine were found by Versalovic *et al.*[5] in *H. pylori* clinical isolates and confirmed by other authors who also found A to C mutation.[6–9]

Xia *et al.*[10] found that nine of the 20 (45%) clarithromycin-resistant *H. pylori* clinical isolates tested reverted to sensitive after two to five subcultures on drug-free agar. However, Hulten *et al.*[8] could not reproduce those results; the seven clarithromycin-resistant *H. pylori* strains remained stably resistant after 50 subcultures *in vitro*.

The aim of this study was to determine the influence of the MIC and the type of mutation in the 23S rRNA on the loss of *in vitro* clarithromycin resistance after several subcultures in the laboratory in 30 clarithromycin-resistant *H. pylori* clinical isolates. The effect of macrolide subinhibitory concentrations in the development of resistance to these antibiotics was also studied.

Materials and methods

Microorganisms

Thirty clarithromycin-resistant *H. pylori* strains were obtained from gastric biopsy samples cultured on selective and non-selective plates. Plates were incubated at 37°C in a microaerobic atmosphere for 7–10 days for primary culture and every 2–3 days for subculture. Strains were identified according to colony morphology, Gram's stain and positive reaction with urease, catalase and oxidase tests, and stored at −80°C in trypticase soy broth containing 20% glycerol until use. TIGR 26695 and NCTC 11638 were used to study the effect of subinhibitory concentrations of macrolides on the development of resistance. The latter strain was also used as a control in the susceptibility tests.

MIC determination

MIC was determined by an agar dilution method. Serial dilutions of the antibiotic ranging from 0.008 to 128 mg/L were prepared in Mueller–Hinton agar supplemented with 10% horse blood. Isolates were grown for 48 h in BHI plus

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10% fetal calf serum, a suspension of 10^9 cfu/mL applied with a Steers replicator and plates were incubated for 3–5 days. Resistance was considered as a clarithromycin MIC ⩾4 mg/L.

Evaluation of stability

The 30 clarithromycin-resistant *H. pylori* clinical isolates were subcultured in drug-free Columbia agar plus 7% blood every 2–3 days 10–20 times and the MIC determined every five passages to detect *in vitro* stability.

Genotypic determination of resistance

H. pylori DNA was extracted by the CTAB reagent method. A2142G and A2143G mutations were determined by the PCR–RFLP method.[5] A 3′-mismatched PCR was used to detect A2142C as described previously.[9]

Effect of subinhibitory concentrations

NCTC 11638 and TIGR 26695 strains were subcultured every 2 days in blood medium containing one of the three macrolides (erythromycin, azithromycin or clarithromycin) at 1/2 × or 1/8 × MIC, for up to 2, 4 or 6 days. Moreover, NCC 11638 was subcultured at 1/2 × MIC for up to 10 and 20 days. Resistance after exposure to subinhibitory concentrations was determined by studying MICs the three macrolides and confirmed by PCR–RFLP to detect A2142G and A2143G mutations in the 23S rRNA gene.

Results

The clarithromycin MICs ranged from 8 to 64 mg/L in the 30 clarithromycin-resistant *H. pylori* strains selected for this study (Table I). Seven of the 30 (23.3%) resistant strains carried the A2142G mutation, three (10%) carried

Table I. Initial and final MICs (mg/L) after the maximum number of subcultures and type of rRNA mutation in the 30 clarithromycin-resistant *Helicobacter pylori* strains studied

Strains	Initial MIC	No. of subcultures	Final MIC	Mutation
1	16	14	8	A2143G
2	8	14	16	A2143G
3	8	18	16	A2143G
4	8	14	32	A2143G
5	16	13	0.064	A2142G
6	8	14	16	A2143G
7	8	15	32	A2143G
8	16	14	16	A2143G
9	8	18	16	A2143G
10	8	15	16	A2143G
11	16	16	16	A2143G
12	8	14	8	A2143G
13	8	18	32	A2143G
14	8	10	0.008	A2143G
15	8	7	16	A2143G
16	64	18	32	A2142G
17	64	13	32	A2142C
18	32	18	0.016	A2142G
19	16	9	16	A2142G
20	32	19	8	A2142G
21	16	13	8	A2143G
22	8	13	16	A2142G
23	8	14	8	A2143G
24	64	11	16	A2142C
25	32	14	16	A2142G
26	32	14	32	A2142C
27	8	16	16	A2143G
28	8	16	16	A2143G
29	8	9	16	A2143G
30	8	8	16	A2143G

the A2142C mutation and 20 (66.6%) the A2143G mutation. Three of the 30 (10%) clarithromycin-resistant strains tested lost their resistance after 10, 13 or 18 subcultures (MIC changed from 8 to 0.008, from 16 to 0.064 and from 32 to 0.016 mg/L). Resistance was unstable in one of 17 strains with MIC 8 mg/L, one of six with MIC 16 mg/L and one of four with MIC 32 mg/L. Resistance was unstable in two of seven strains with the A2142G mutation and in one of 20 strains with the A2143G mutation.

When the effect of subinhibitory concentrations was studied, no significant changes in the macrolide MIC values were observed at either 1/2 × or 1/8 × MIC for up to 10 days. The results are shown in Table II. A change in the MIC was observed only when NCTC 11638 was exposed to 0.25 mg/L (1/2 × MIC) of erythromycin for 20 days. A genomic study of the colonies showed an A2143G mutation in this strain. This effect was not observed with clarithromycin or azithromycin.

Discussion

Infection by clarithromycin-resistant strains is correlated with lower eradication rates, although in some patients eradication is achieved, in spite of them being infected by clarithromycin-resistant strains. The presence of unstable resistance *in vivo* could explain the efficacy of the antibiotic in such cases.

According to our data, clarithromycin resistance was unstable in three of the 30 (10%) *H. pylori* strains. When other authors studied stability they found different results. Whilst Xia *et al.*[10] found that 45% of strains (9 out of 20) showed unstable resistance after two to five subcultures, Hulten *et al.*[8] found that in all the seven strains tested resistance was stable after as many as 50 subcultures. Debets-Ossenkopp *et al.*,[6] studying 20 clinical isolates, found that after 21 passages in clarithromycin-free medium all strains remain resistant.[6] In our study, we needed more than the two to five subcultures described by Xia *et al.*, but we found that resistance was not stable after 10, 13 or 18 subcultures.

We found that the MIC for most of our strains was 8 mg/L (17 strains) compared with 16 mg/L for six strains, 32 mg/L for four strains and 64 mg/L for three strains. We observed that only one of the 8 mg/L group lost resistance (5.8%) compared with one of six in the 16 mg/L group (16.6%) or one of four with an MIC of 32 mg/L (25%). In contrast, Xia *et al.*[10] observed that isolates for which the MICs were ⩽32 mg/L were more likely to revert to susceptible (6/9), than those for which they were >32 mg/L (3/11).

Most of our clarithromycin-resistant strains had an A2143G mutation (20 strains), seven had A2142G and three had A2142C. In two of the seven strains with the A2142G mutation, resistance was unstable (28.5%) compared with only one of 20 with A2143G mutation (5%) or none out of three with A2142C. Hulten *et al.*[8] studied four strains with the A2142G mutation and two with A2143G and they found no loss of resistance. In the study performed by Debets-Ossenkopp *et al.*,[6] 13 strains with A2142G and seven with A2143G lost no resistance.

When NCTC 11638 and TIGR 26695 were exposed to subinhibitory concentrations of macrolides no changes were observed after 10 days' exposure to clarithromycin, erythromycin or azithromycin. However, cross-resistance to the three macrolides was obtained after 20 days' exposure to erythromycin but not to clarithromycin or azithromycin.

The reversibility of clarithromycin resistance could have at least two clinical consequences: (i) clarithromycin may eradicate *H. pylori*, in spite of apparent resistance *in vitro* due to the instability of the resistance; (ii) the clarithromycin resistance rate *in vitro* may be underestimated due to the subcultures performed from the first isolation to the moment of performing the susceptibility testing. However, the clinical importance of these data is at the moment unknown and further studies are needed.

Table II. Effect on the MIC (mg/mL) of erythromycin (E), azithromycin (A) and clarithromycin (C) after exposure of *Helicobacter pylori* to different subinhibitory concentrations of macrolides for a varying number of days

				MIC (mg/mL)		
Strain	Macrolide	Days	Concentration	erythromycin	azithromycin	clarithromycin
TIGR 26695	control	–	–	0.125	0.125–0.25	0.032–0.064
	E, A or C	2, 4 or 6	1/2 or 1/8 MIC	0.064–0.125	0.125–0.25	0.032–0.064
NCTC 11638	control	–	–	0.5	0.5	0.064–0.125
	E, A or C	2, 4 or 6	1/2 or 1/8 × MIC	0.25–0.5	0.25–0.5	0.032–0.125
	E, A or C	10	1/2 MIC	0.064–0.25	0.125–0.5	0.016–0.125
	A or C	20	1/2 MIC	1–2	1–2	0.25
	E	20	1/2 MIC	64	64	16

Control indicates the MIC value without exposure to macrolides.

In summary, 10% of the clarithromycin-resistant strains studied here lost their resistance after 10–18 subcultures *in vitro*. A very long exposure of NCTC 11638 to a sub-inhibitory concentration of erythromycin led to the development of resistance to all macrolides *in vitro*. However, this effect was not observed with other macrolides and for shorter periods of exposure.

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