Simultaneous colonisation of *Helicobacter pylori* with and without mutations in the 23S rRNA gene in patients with no history of clarithromycin exposure

M Matsuoka, Y Yoshida, K Hayakawa, S Fukuchi, K Sugano

Abstract

Background—It was recently reported that A to G transition mutations at positions 2143 and 2144 in the 23S rRNA gene are associated with clarithromycin resistance in *Helicobacter pylori*.

Aims—To study the incidence and mechanism of development of clarithromycin resistance by analysing these mutations.

Subjects—Eighty two *H pylori* positive patients who had an endoscopic examination and no history of treatment with macrolide antibiotics.

Methods—Clarithromycin resistance was screened for by polymerase chain reaction-restriction fragment length polymorphism of the 23S rRNA gene coupled with antibiotic susceptibility testing. In clinical isolates with mutations or resistance, mutations in individual colonies were analysed by direct sequencing.

Results—Of the 79 amplicons (DNA fragments amplified by polymerase chain reaction), *Alw26I* and *MboII* digestion disclosed the mutation in four (5%) and one (1%) respectively. However, the *Alw26I* cleavage was incomplete in two of the four amplicons, as was the *MboII* cleavage. Individual colony analysis of the isolates with incomplete cleavage patterns showed the presence of both wild type and mutated strains in the 23S rRNA genes.

Conclusions—Both clarithromycin sensitive and resistant strains colonised in some patients with no history of exposure to macrolides. The results suggest that resistant strains may not be formed but selected by clarithromycin administration.

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Keywords: *Helicobacter pylori*; clarithromycin resistance; 23S rRNA gene; point mutation; mixed strain colonisation

Clarithromycin is one of the most useful antibiotics in eradication of *Helicobacter pylori*. However, several reports have shown that about 1–10% of *H pylori* isolates are resistant to clarithromycin,^{1–5} which may have resulted in the failure of such treatment.^{6–8} Therefore it is important to examine clarithromycin resistance when considering eradication. Versalovic *et al*⁹ reported that A2143G and A2144G mutations in domain V of the 23S rRNA genes of *H pylori*, cognate with residues 2058 and

Table 1 Diagnosis and sex of the subjects

Diagnosis	Male	Female	Total
Gastric ulcer	16	11	27
Duodenal ulcer	13	6	19
Combined ulcer	13	1	14
Gastric cancer	4	1	5
Other	14	3	17
Total	60	22	82

2059 in *Escherichia coli*, are associated with clarithromycin resistance. We therefore studied these mutations in *H pylori* isolates by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and direct sequencing in patients with no history of clarithromycin exposure to determine whether clarithromycin resistant strains are formed or merely selected by clarithromycin treatment.

Subjects and methods

SUBJECTS

The subjects comprised 82 patients who were having endoscopic examinations for upper gastrointestinal problems and had no history of treatment with macrolide antibiotics (table 1). Previous antibiotic use was excluded by examining their medical records closely and carrying out a detailed interview. Patients whose history of previous medical treatment or antibiotic use was not certain were excluded. Written informed consent was obtained from the patients and the study was approved by the institution's ethical committee.

BACTERIOLOGY

H pylori status was examined by conventional culture and histological tests (Giemsa stain). Four biopsy specimens from different parts of the stomach were obtained to detect *H pylori* infection. *H pylori* were isolated from gastric biopsy specimens by culturing on Columbia blood agar base (Oxoid, Basingstoke, Hampshire, UK) containing Skirrow's selective supplement (Oxoid) and 7% fresh horse blood under microaerobic conditions. Clarithromycin sensitivity was evaluated by the agar dilution test. Colony formation at or above a drug concentration of 5 mg/l was judged as resistant.

PCR METHOD

Separate gastric biopsy specimens (65 patients) were obtained for DNA extraction using a QIAamp tissue kit (Qiagen, Hilden, Germany).

Abbreviations used in this paper: PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism.

Department of
Gastroenterology,
Mishuku Hospital,
5-33-12 Kamimeguro,
Meguro-ku, Tokyo,
Japan 153

M Matsuoka
Y Yoshida
K Hayakawa
S Fukuchi

Health Service Center,
University of Tokyo,
3-8-1 Komaba,
Meguro-ku, Tokyo
K Sugano

Correspondence to:
Dr M Matsuoka.

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Figure 1 Representative polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) pattern of 23S rRNA genes from patients with clarithromycin resistance. Lane M, DNA size marker; lane A, PCR-RFLP pattern obtained with *Mbo*II; lane B, PCR-RFLP pattern produced by *Alw*26I. Case 1-A, Three bands corresponding to the predicted 301, 192, and 109 bp fragments were detected. The amplicon digested by *Mbo*II gave an incomplete digestion pattern. Case 1-B, Two bands with predicted sizes (265 and 36 bp) were detected. The amplicon without mutation at 2144G gave this pattern. Case 2-A, One 301 bp band was detected. The amplicon without mutation at 2143G gave this pattern. Case 2-B, The amplicon with a mutation at A2144G was completely digested by *Alw*26I, producing the predicted 209, 56, and 36 bp fragments. Case 3-A, The pattern was similar to that of case 2-A. Case 3-B, Four bands corresponding in size to 265, 209, 56, and 36 bp were detected, showing an incomplete digestion pattern.

DNA samples were also prepared from 17 *H. pylori* isolates as described above. We designed primers using the 23S rRNA gene sequence reported by Hiratsuka (Genebank accession number U27270). The primers were as follows: forward primer, 5'-CTGCATGAATGGCG TAACGAG-3' (complementary to 23S rRNA gene sequence from 2047 to 2067), and reverse primer, 5'-GAGCGACCGCCCCAGTCAAA C-3' (complementary to 23S rRNA gene sequence from 2327 to 2347), which will generate a 301 bp product. PCR amplifications were performed with Ex-Taq polymerase (Takara, Tokyo, Japan) and a model TP240 PCR thermal cycler (Takara). The amplification reaction mixture (50 µl) contained 2.0 mM MgCl₂, 1.0 U of Ex-Taq polymerase, and 0.5 µl of the sample DNA. Cycling conditions were 30 cycles of 94°C for one minute, 56°C for 30 seconds, 68°C for 30 seconds, followed by a final extension step at 68°C for seven minutes. The PCR product was resolved on an 8% polyacrylamide gel and the gel was stained with SYBER Green I nucleic acid gel stain (Molecular Probes, Eugene, Oregon, USA). In order to evaluate our PCR method, we also performed the established PCR method targeting urease A.¹⁰

MUTATION ANALYSIS

We analysed mutations in the 23S rRNA gene of *H. pylori* by RFLP. Amplicons (PCR amplified DNA fragments) were digested by the restriction enzymes, *Mbo*II (Takara) and *Alw*26I (Fermentas, Vilnius, Lithuania). Mutations create an additional site for digestion by these restriction enzymes: *Mbo*II for the A2143G mutation and *Alw*26I for the A2144G mutation. Reaction mixtures (20 µl) that contained 6.0 U *Mbo*II or 5.0 U *Alw*26I, 10 µl

of the PCR product, 2 µl digestion buffer, and 8 µl water were incubated at 37°C for one hour.

In the case of *H. pylori* isolates with mutations or resistance, DNA samples extracted from each colony were amplified by PCR and analysed for the mutations by RFLP as described above and also by direct sequencing. PCR products were purified with a QIAquick PCR purification kit (Qiagen) followed by cycle sequencing with ABI Prism dRhodamine terminator cycle sequencing ready reaction kit (Applied Biosystems, Foster City, California, USA) according to the manufacturer's protocol. Extension products were purified by ethanol precipitation. Sequence analysis was performed with a genetic analyser (ABI Prism 310; Applied Biosystems).

Results

PCR AMPLIFICATION OF THE 23S rRNA GENE

We successfully amplified the expected 301 bp fragment in 96% (79/82) of samples; 62 of 65 DNA samples were extracted directly from gastric biopsy specimens and 17 of 17 from *H. pylori* colonies. In two of the three *H. pylori* positive patients in which we failed to amplify the PCR product, the urease A gene was also not amplified. In contrast, in six patients judged as *H. pylori* negative by conventional methods, we could amplify the target DNA from gastric biopsy specimens, whereas only one was positive by PCR directed to urease A (data not shown).

DETECTION OF 23S rRNA GENE MUTATIONS BY PCR-RFLP

In these 79 samples, we analysed mutations of the 23S rRNA gene of *H. pylori* by RFLP. Of the 79 amplicons, four (5%) gave a mutated RFLP pattern after *Alw*26I digestion and one (1%) after *Mbo*II digestion. Two of the four amplicons (nos 49 and 71) were completely cleaved by *Alw*26I, but the cleavage was incomplete in the other two (nos 51 and 82). The one amplicon digested by *Mbo*II also gave an incomplete cleavage pattern (fig 1).

COMPARISON BETWEEN MUTATIONS AND SUSCEPTIBILITY TO CLARITHROMYCIN

We evaluated clarithromycin susceptibility by agar dilution test in 41 samples. All four isolates with the *Alw*26I cleavage site were clarithromycin resistant. The one with the *Mbo*II cleavage site, however, was clarithromycin sensitive by the agar dilution test. The rest of the isolates resistant to either *Alw*26I or *Mbo*II digestion were clarithromycin sensitive except for one (no 75).

DETECTION OF THE MUTATIONS FROM EACH COLONY BY PCR-RFLP AND DIRECT SEQUENCING

In isolates nos 51 and 82 in which *Alw*26I digestion was incomplete, RFLP patterns varied depending on the individual colony: some colonies showed complete digestion patterns whereas an incomplete pattern was seen in others. In no 82, colonies with a wild type pattern were also detected. Isolates nos 49 and 71, both showing a complete cleavage pattern on digestion with *Alw*26I, also contained heterogeneous colonies, although the cleavage

Table 2 Polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) patterns from different colonies

Isolate no	Patient		Restriction enzyme	RFLP pattern	RFLP pattern (from each colony)			Sensitivity to clarithromycin
	Age	Sex			Complete	Incomplete	No mutation	
49	42	M	<i>Alw26I</i>	Complete	10	2	0	Resistant
71	53	M	<i>Alw26I</i>	Complete	12	4	0	Resistant
51	46	M	<i>Alw26I</i>	Incomplete	7	8	0	Resistant
82	44	M	<i>Alw26I</i>	Incomplete	10	7	2	Resistant
8	63	M	<i>MboII</i>	Incomplete	0	4	5	Sensitive
75	47	M	<i>MboII</i>	—	0	1	12	Resistant

Table 3 Direct sequence from different colonies

Isolate no	Patient		Restriction enzyme	RFLP pattern	Direct sequence (from each colony) A to G mutation			Sensitivity to clarithromycin
	Age	Sex			2143	2144	No mutation	
49	42	M	<i>Alw26I</i>	Complete	0	4	0	Resistant
71	53	M	<i>Alw26I</i>	Complete	0	7	0	Resistant
51	46	M	<i>Alw26I</i>	Incomplete	0	4	2	Resistant
82	44	M	<i>Alw26I</i>	Incomplete	0	6	4	Resistant
8	63	M	<i>MboII</i>	Incomplete	3	0	3	Sensitive
75	47	M	<i>MboII</i>	—	4	0	2	Resistant

RFLP, restriction fragment length polymorphism.

was complete in most of them. In isolate no 8, which had an incomplete pattern on *MboII* digestion, an incomplete pattern was shown in four colonies whereas five colonies showed the wild type pattern. In isolate no 75, which showed clarithromycin resistance despite a normal RFLP pattern, colony analysis disclosed a single colony with an *MboII* cleavage site (table 2).

In isolates nos 49 and 71, all colonies were shown to have an A2144G transition mutation by sequence analysis. In isolates nos 51 and 82, colonies with an A2144G mutation and those without any mutation were present. In isolate nos 8 and 75, mixed colonies with an A2143G mutation and those without mutation were detected (table 3). In any event, sequence analysis on individual colonies showed that the mutations involved both copies of the 23S rRNA gene simultaneously, excluding the possibility that one of the two copies of the 23S rRNA gene is mutated (data not shown). In addition to these mutations, we found others, such as T2183C and T2245C, in many isolates from these Japanese patients. However, these mutations were found mutually in both sensitive and resistant colonies (data not shown).

Discussion

Clarithromycin is one of the most often used and important antibiotics in eradication treatment for *H pylori*. As the presence of clarithromycin resistant *H pylori* may result in eradication failure, it is very important to predict bacterial resistance and establish an accurate susceptibility testing method. Clarithromycin inhibits protein synthesis by binding to the peptidyltransferase loop of 23S rRNA,¹¹ which has been shown at residues A2058 and A2059 in the 23S rRNA gene of *E coli*.¹² When these residues mutate, the affinity of clarithromycin binding to ribosomes is reduced, resulting in the clarithromycin resistance. Versalovic *et al*⁹ have reported that, in *H pylori*, transitional mutations (A2143G and A2144G, cognate with *E coli* 23S rRNA positions 2058 and 2059) were associated with clarithromycin

resistance. Several reports confirmed that the same mutations are responsible for the resistance.^{13–18}

We could detect *H pylori* infection by amplifying the 23S rRNA gene of *H pylori* using Ex-*Taq* polymerase in patients with positive *H pylori* status, except for a few cases. The negative cases may have resulted from sampling errors, because in two thirds of them PCR amplifications targeting urease A also failed. In contrast, we obtained positive amplifications in several *H pylori* negative patients by conventional methods. Therefore this PCR method employing Ex-*Taq* polymerase, which has higher fidelity and efficiency of amplification than conventional *Taq* polymerases, seems to have comparable or better sensitivity and specificity for the diagnosis of *H pylori* than a combination of culture and histological methods.

In addition to its diagnostic merit, it allows us to examine by RFLP and direct sequencing the molecular structure of the 23S rRNA gene that is essential for the macrolide resistance. Of the 79 samples, four (5%) were digested by *Alw26I* and one (1%) by *MboII*. This mutation rate was similar to the detection rate of resistant strains reported in other countries.^{1–5} We could not detect the rather rare A2143C mutation, recently reported by Stone *et al* and Occhialini *et al*.^{14–16–18} In two of the four amplicons digested by *Alw26I* and the one by *MboII*, cleavage was not complete, leaving some of the original amplicons undigested. Based on these mixed patterns of RFLP, we suspected that these patients may have been infected with either a mixed population of mutant (clarithromycin resistant) and wild type strains of *H pylori* or a strain with heterozygosity of the 23S rRNA gene. In order to examine these possibilities, we analysed in *H pylori* isolates with mutations or resistance the PCR product from each colony by PCR-RFLP and direct sequence. In isolates that were completely digested with *Alw26I*, most colonies were shown by RFLP analysis to have an A2144G transition mutation, although some of them

showed a mixed pattern. In isolates incompletely digested by *Alw26I* or *MboII*, colonies with the A to G transition mutation at 2143 or 2144 and colonies without mutations were found. Sequence analysis of amplicons from individual colonies confirmed the RFLP results. These results suggest that, in each patient, heterogeneous strains with various genotypes form colonies simultaneously. In metronidazole resistance, co-infection with a population of metronidazole sensitive and resistant strains has been suggested.^{19, 20} Although Versalovic *et al*⁹ have reported on one isolate with heterozygosity of the 23S rRNA gene, our detailed colony analysis showed that almost all the mutations were homozygous for the 23S rRNA gene. In addition, we detected several other mutations such as T2183C and T2245C, which were similarly found in both sensitive and resistant colonies, and hence may represent a local variation of Japanese strains, unrelated to drug resistance.

We found some discrepancy between mutational analysis and susceptibility testing to clarithromycin: all the isolates with an *Alw26I* digestion pattern were clarithromycin resistant, but the one digested by *MboII* was clarithromycin sensitive. In addition, we found one isolate that showed resistance despite having a normal RFLP pattern, although detailed colony analysis disclosed the presence of a small number of resistant strains in this case. Thus the results of mutational analysis correlated well with susceptibility testing in most cases, but it should be noted that some cases may give discordant results when the ratio between the heterogeneous strains is greatly skewed.

We think that our PCR method can offer an accurate and quick way of not only diagnosing *H. pylori* infection but also identifying clarithromycin resistant strains. Although macrolide use may not have been reported accurately by the patients, our data showing the presence of both mutant and wild type *H. pylori* strains in these patients strongly suggest that they were indeed not previously exposed to macrolide antibiotics, because the mutations were fairly stable and not easily reversed.¹⁷ Spontaneous mutation of a single base is known to occur at a relatively constant rate of 10^{-9} /base/cell division. Considering the reported genome size (1.6×10^6 bases/haploid) of *H. pylori*,²¹ the probability of spontaneous mutation occurring in a single bacterial genome is estimated to be $10^{-9} \times 1.6 \times 10^6 \times 2$. As most of the clarithromycin resistance is caused by point mutation at 2143 and 2144, the probable spontaneous mutation rate at these sites is calculated to be 4.0×10^{-9} /cell division. As the number of *H. pylori* is estimated to be 10^7 – 10^9 /100 μ l gastric mucus,^{22, 23} the total number of bacteria in a stomach may be in the range 10^9 – 10^{11} , assuming the amount of gastric mucus is limited to 10 ml. In addition to those in the mucus, a similar number of bacteria adhere to the mucosa, therefore the total number would be twice that. Therefore it is quite reasonable to assume that between eight and 8×10^2 resistant bacteria emerge after each cell

division. Although we do not know exactly how *H. pylori* multiplies in the stomach, the chance of creating a clarithromycin resistant strain with a mutation at either of these two bases should be much higher, as they are not quiescent and will repeat cell division. The mutagenic effect of nitric oxide or an incomplete mismatch repair system in *H. pylori*²¹ may further increase the mutation rate. Thus it is possible that a small number of clarithromycin resistant strains may emerge spontaneously in the stomach. As they are only a minority of the population, it would be difficult to detect such a small number of mutant bacteria. However, after incidental clarithromycin exposure, these resistant strains will survive and become dominant enough to be detectable by conventional tests. This would explain the apparent increase in clarithromycin resistance. In conclusion, our results suggest that the apparent bacterial resistance to clarithromycin may not be induced but actually result from selection and enrichment of resistant strains by antimicrobial treatment.

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