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Point mutations in the 23S rRNA gene of *Helicobacter pylori* associated with different levels of clarithromycin resistance

James Versalovic[a]*, Michael S. Osato[a], Klaudia Spakovsky[a], Maria Pina Dore[a], Rita Reddy[a], Gregory G. Stone[b], Dee Shortridge[b], Robert K. Flamm[b], S. Ken Tanaka[b] and David Y. Graham[a]

[a]*Digestive Diseases, Veterans Affairs Medical Center, 2002 Holcombe Boulevard, Houston, TX 77030;* [b]*Anti-Infective Division, Abbott Laboratories, 100 Abbott Park Road, Abbott Park, IL 60064, USA*

Fifty-four of 59 (91.5%) clarithromycin-resistant isolates of *Helicobacter pylori* from different patients possessed either the A2143G (formerly A2058G) or the A2144G (formerly A2059G) mutation in the gene encoding 23S rRNA. The A2143G mutation was significantly more likely to occur in isolates with MICs exceeding 64 mg/L (65% versus 30% with the A2144G mutation; *P* = 0.01). The majority (26 of 31; 83.9%) of isolates with the A2143G mutation had MICs exceeding 64 mg/L. Peptic ulcer disease recurred in a substantial proportion of patients infected with *H. pylori* strains containing either the A2143G or the A2144G mutation.

Introduction

Isolation of clarithromycin-resistant *Helicobacter pylori* has occurred with increased frequency in patients who have failed treatment regimens.[1–3] Recently, mutations in the 23S rRNA gene of *H. pylori* isolates obtained from patients with gastroduodenal disease were associated with clarithromycin resistance.[4,5] Two mutations were originally described as A2058G or A2059G based on nucleotide substitutions at positions cognate with numbered *Escherichia coli* 23S rRNA residues. The recent availability of the entire *H. pylori* 23S rRNA sequence (GenBank accession no. U272270) led to the names of the mutations being changed to A2143G (formerly A2058G) and A2144G (formerly A2059G) to conform with recently established *H. pylori* sequence coordinates.

In order to evaluate the relative prevalence of each mutation among clarithromycin-resistant *H. pylori*, a larger study was conducted with 59 isolates from different patients.

Patients and methods

Patients

Fifty-nine *H. pylori* isolates were cultured from gastric antral biopsies obtained from 59 different patients participating in clinical trials sponsored by Abbott Laboratories. Patients with duodenal ulcer disease confirmed by endoscopic evaluation were examined at different localities in the United States and Canada and treated as described previously.[4]

Microbiological culture conditions

H. pylori isolates were passaged routinely on brain heart infusion (BHI) agar containing 7% fresh horse blood and incubated at 37°C in 12% CO_2 and 98% humidity. *H. pylori* was presumptively identified on the basis of colony morphology. Microscopy confirmed the expected Gram-negative, curved morphology. Isolates were verified biochemically as *H. pylori* by positive reactions for catalase, oxidase and urease activity.

Antimicrobial susceptibilities

Etest (AB Biodisk, Solna, Sweden) determinations of MICs were performed on Mueller–Hinton agar (150 mm) plates (Difco Laboratories, Detroit, MI, USA) supplemented with 5% sheep blood (BBL Microbiology Systems, Cockeysville, MD, USA). A sterile swab was dipped into a bacterial suspension equivalent to a no. 2 McFarland standard. After swabbing the entire plate surface with the inoculum, sterile Etest strips impregnated with clarithromcyin ranging in concentration from 0.016 to

*Corresponding author. Present address: Department of Pathology, Massachusetts General Hospital, Boston, MA 02114, USA. Tel: +1-617-726-5977; Fax: +1-617-726-3256; E-mail: jversal@tiac.net

256 mg/L were placed on the agar surface. Plates were incubated at 37°C, 12% CO_2, 98% humidity for 3–4 days. MICs were determined as described previously. Isolates were classified as resistant to clarithromycin if the MIC exceeded 2 mg/L[6] on the basis of established National Committee of Clinical Laboratory Standards (NCCLS) criteria.[7]

Cell lysate preparations

Cells were cultivated on BHI agar plates until confluent growth had been reached. Cells were subjected to detergent lysis under alkaline conditions. Briefly, a loopful of cells was added to 20 μL of lysis buffer (0.05 M sodium hydroxide, 0.25% sodium dodecyl sulphate) and incubated at 95°C for 15 min. After heating, 200 μL of MilliQ water was added to dilute the cells prior to centrifugation at 12,000**g** for 5 min. Two microlitres of supernatant from these crude lysates were added to each PCR amplification reaction.

Detection of mutations

Point mutations were identified by PCR amplification of the target genes encoding 23S rRNA and subsequent digestion of the 1402 bp PCR product to produce differently sized DNA fragments if mutations were present. PCR amplifications using crude cell lysates (instead of purified chromosomal DNA) were performed as previously described[4] with oligonucleotide primers 18 and 21,[4,8] complementary to conserved regions of the genes encoding 23S rRNA. Restriction digestions with *Bsa*I[4] or *Mbo*II were performed with 23S rRNA PCR products. For detection of the A2144G mutation, 5 U of *Bsa*I (New England Biolabs, Beverly, MA, USA) were added to each amplicon and digestions were performed for 14 h at 55°C as described previously.[4] Diagnosis of the A2143G mutation required incubation with 7.5 U of *Mbo*II (New England Biolabs) for 14 h at 37°C. The presence of the A2143G mutation creates an *Mbo*II site (wild type $AAAGA(N)_8 \rightarrow$ mutant $GAAGA(N)_8$) in the 23S rRNA gene amplicon and yields digestion products of 710 and 692 bp that comigrate in agarose gels. Digested PCR products were analysed by electrophoresis within a 1% agarose gel in Tris–acetate–EDTA (TAE) buffer and ethidium bromide (0.5 mg/L).

Results and discussion

Either the A2143G or the A2144G mutation was present in 91.4% (54 of 59) of clarithromycin-resistant *H. pylori* isolates examined (Table). A subset of isolates with either the A2143G or A2144G mutation are depicted following *Mbo*II digestion in the Figure. As previously demonstrated,[4] the A2144G mutation was detected similarly by *Bsa*I digestion. The majority of isolates (52.5%) contained the A2143G mutation, whereas the A2144G mutation was present in 39% of isolates. These data extended the initial analysis of 23S rRNA mutations in *H. pylori*[4] in which we described the A2143G (A2058G) or A2144G (A2059G) mutations in isolates from seven patients. In the present study, five (of 59) isolates lacked either mutation and the basis for clarithromycin resistance remains undetermined. As a third mutation has been recently described in

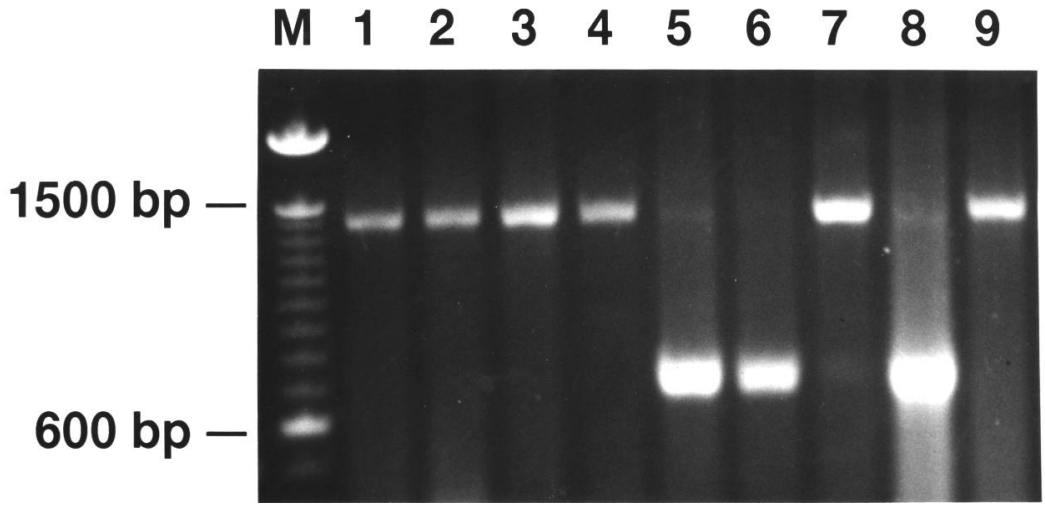

Figure. Detection of the A2143G mutation in 23S rRNA genes by *Mbo*II digestion of PCR products. The lane marked M contains a 100 bp DNA ladder that serves as the size marker (Gibco BRL, Gaithersburg, MD, USA). Sample lanes are as follows: lane 1, patient A, MIC = 256 mg/L, A2144G mutation; lane 2, patient B, MIC = 16 mg/L, A2144G mutation; lane 3, patient C, MIC = 256 mg/L, A2144G mutation; lane 4, patient D, MIC = 4 mg/L, A2144G mutation; lane 5, patient E, MIC = 256 mg/L, A2143G mutation; lane 6, patient F, MIC > 256 mg/L, A2143G mutation; lane 7, patient G, MIC = 128 mg/L, A2144G mutation; lane 8, patient H, MIC = 64 mg/L, A2143G mutation; lane 9, patient I, MIC = 4 mg/L, undetermined mutation. Note that presence of the A2144G mutation could not be detected in this assay, but required further digestion of PCR products with *Bsa*I.

Table. Association between clarithromycin MICs and 23S rRNA mutations in *H. pylori*

Mutation	Number (%) of isolates MIC ⩽ 64 mg/L (n = 19)	MIC > 64 mg/L (n = 40)	Total (n = 59)
A2143G	5 (26.3)	26 (65.0)	31 (52.5)
A2144G	11 (57.9)	12 (30.0)	23 (38.9)
Undetermined	3 (15.8)	2 (5.0)	5 (8.5)

H. pylori,[9] DNA sequencing of the entire 23S rRNA gene or other genes encoding ribosomal RNA or proteins may yield different mutations in these isolates.

MICs were determined by Etests[6] and correlated with the presence of either 23S rRNA gene mutation (Table). Excellent concordance was found previously between clarithromycin MICs obtained by Etests, microbroth dilution assays and disc diffusion.[6] When an MIC of >64 mg/L was used to define high-level clarithromycin resistance, differences in the prevalence of each mutation with respect to MIC were observed. Twenty-six (83.9%) isolates with the A2143G mutation demonstrated high-level resistance and the majority (65%) of isolates with MICs exceeding 64 mg/L contained the A2143G mutation. In contrast, most (57.9%) isolates with MICs of ⩽64 mg/L contained the A2144G mutation. If an MIC of 32 mg/L was used to define high-level resistance, 96.8% (30 of 31) of isolates with the A2143G mutation demonstrated high-level resistance.

More than 90% of clarithromycin-resistant *H. pylori* isolates contained either of two transition mutations in a conserved domain of genes encoding 23S rRNA. Such a preponderance of mutations in this sample supports the usefulness of the Etest for susceptibility testing of *H. pylori.* High-level resistance to clarithromycin is associated with the presence of the A2143G mutation in *H. pylori*. The presence of the A2143G mutation in a population of *H. pylori* infecting the gastric mucosa may signify a greater probability of treatment failure and disease recurrence following macrolide-based treatment regimens. Thirty-seven of 54 patients with *H. pylori* isolates containing the A2143G or A2144G mutations had documented and available follow-up evaluation by oesophagogastroduodenoscopy (OGD). In this subset, ulcer disease recurrence was documented by OGD in 52% (13 of 25) of patients with isolates containing the A2143G mutation and 33% (four of 12) of patients with isolates containing the A2144G mutation.

This report demonstrates the correlation of the A2143G mutation with high-level clarithromycin resistance. The rapid *H. pylori* cell lysis and mutation assessment presented in this report permits consideration of 24 h molecular antimicrobial susceptibility testing directly from a gastroduodenal biopsy. The presence of either mutation, especially the A2143G mutation, may prompt the physician to consider a high-dose, non-macrolide-based treatment regimen.

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