Novel Genotypes in *Helicobacter pylori* Involving Domain V of the 23S rRNA Gene

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Abstract

Background: *Helicobacter pylori* is a pathogenic bacterium that infects a half of the human population. In Chile, between 55% and 79% of people are colonized by *H. pylori*. At present, therapeutic strategies to eradicate the bacterium depend on the knowledge of its resistance to antibiotics. The clarithromycin resistance in *H. pylori* is associated with point mutations in the 23S rRNA. This study analyzes 23S rRNA gene mutations and minimum inhibitory concentration (MIC) for clarithromycin in *H. pylori* isolates from patients of the metropolitan region of Chile.

Materials and methods: *H. pylori* isolates from 50 dyspeptic patients with no history of clarithromycin exposure were tested for clarithromycin resistance by agar dilution method. Resistant strains were analyzed for mutations in the 23S rRNA gene by polymerase chain reaction-based restriction fragment length polymorphism and sequencing.

Results: Primary resistance was observed in 10 isolates (20%). A single mutation was detected in four of the 10 isolates and two or more mutations in the other six cases. The C2147G transversion and G1939A, T1942C, and A2142G transitions in the peptidyltransferase region of domain V were novel.

Conclusions: The study shows: 1, novel variants of the *H. pylori* 23S rRNA gene; and 2, a high prevalence of *H. pylori* displaying primary clarithromycin resistance with low level of MIC in an urban area of the Metropolitan Region of Chile.

Clarithromycin is a macrolide that has been widely used in combination with other antimicrobial agents in association with a proton pump inhibitor in triple-therapy regimens for the eradication of *Helicobacter pylori* infection [1–3]. The antibacterial activity of clarithromycin has been associated to its ability to inhibit protein synthesis by binding to the 50S ribosomal subunit of microorganisms [4]. Previous studies examining clarithromycin-resistant *H. pylori* isolates have revealed that point mutations in the peptidyltransferase region encoded in domain V of the 23S rRNA gene are associated to the mechanism of clarithromycin resistance [5,6]. These mutations abrogate the clarithromycin-binding site in the bacterial ribosome. The two most common mutations associated with clarithromycin resistance are A→G transitions at positions 2142 and 2143 of the 23S rRNA gene [5,7,8]. Other less frequent mutations, such as A2142C, A2115G, G2141A, and T2182C, have been also associated to clarithromycin resistance [7,9–11].

In Chile, studies concerning the frequency of clarithromycin-resistant *H. pylori* isolates are restricted to the VIII region [12]. The aims of the present study were: 1, to characterize the 23S rRNA gene mutations of resistant *H. pylori* isolates by polymerase chain reaction-based restriction fragment length polymorphism (PCR-RFLP) and 2, to evaluate the range of sensibility to clarithromycin in isolates from the Metropolitan Region of Chile.

Materials and Methods

Patients

Sixty-five urease-positive patients with no history of previous triple therapy and subjected to endoscopy between October 2002 and September 2005 at the Gastroenterology Unit-Clinical Hospital of the University of Chile, were enrolled into the study. A written informed consent was obtained from all of them. Three biopsies, two from the antrum and one from the corpus, were obtained from each patient. One of the antrum specimens was used for the rapid urease test and the other two were used for bacterial culture and antibiotic susceptibility testing. *H. pylori* isolates were recovered from 50 patients of the study population.

Bacterial Culture and Antibiotic Susceptibility Testing

Fresh and frozen homogenates of biopsies were streaked onto clarithromycin-free Trypticase Soy agar plates supplemented with 5% horse serum, culture supplement Vitox (Oxoid, Barsingstoke, Hampshire, England), and antibiotic supplement Dent (Oxoid) (TSA agar) [13] and incubated for 3–5 days. *H. pylori* colonies were identified by standard methods [14]. To evaluate antibiotic resistance, 10 colonies from the resulting growing cells were randomly selected and streaked onto TSA agar plates to grow. From each of those cultures, suspensions in phosphate-buffered saline and series of 10-fold dilutions were prepared. Ten-microliter aliquots of each dilution were spotted on freshly prepared TSA agar plates containing varying concentrations of clarithromycin (0, 0.2, 0.5, 1.0, 1.25, 1.5, 1.75, 2.0, 4.0, and 8.0 μg/mL) following the National Committee for Clinical Laboratory Standards (NCCLS) guidelines [3,15,16]. A strain was considered to be susceptible to a concentration of clarithromycin when it decreased the efficiency of colony formation for at least 10-fold.

DNA Extraction and PCR Amplification

DNA extraction from cultured isolates was performed using the Wizard Genomic DNA Purification kit (Promega, Madison, WI, USA). The conserved 1402 base pair (bp) region of the *H. pylori* 23S rRNA gene between nucleotide positions 1445 and 2846 (GenBank accession no. U27270) was amplified using forward primer 5′-AGTCGGGGACCTAAGGCGAG-3′ and reverse primer 5′-TTCCCGCTTAGATGCTTTCAG-3′ [16]. PCR conditions were: one cycle at 98 °C for 3 minutes followed by 30 cycles at 98 °C for 1 minute, 54 °C for 1 minute, and 74 °C for 1 minute. A final elongation step was at 74 °C for 15 minutes, using ThermoAce™ DNA Polymerase (Invitrogen, Carlsbad, CA, USA). The procedure was performed in a PTC-100 MJ Research thermal cycler. Amplified fragments were visualized on a 1% agarose gel electrophoresis stained with ethidium bromide.

RFLP Analysis

To detect the A→G point mutation at nucleotide position 2142, PCR products were digested with the *Mbo*II (5′...GAAGA $(N)_8^{\nabla}$...3′) or *Bbs*I (5′...GAAGAC $(N)_2^{\nabla}$...3′) restriction enzymes (BioLab, Beverly, MA, USA). To detect mutations at position 2143, PCR products were digested with *Bsa*I (5′...GGTCTC $(N)_1^{\nabla}$...3′) (BioLab) and analyzed on 1% agarose gels. *Mbo*II- and *Bbs*I-digested PCR products containing the A2142G mutation are expected to yield two fragments (710–692 bp and 716–686 bp, respectively) (Fig. 1A1,1B1), *Bsa*I-digested PCR products containing the A2143G mutation are expected to display three fragments (395, 314, and 693 bp) (Fig. 1C1). The control strain was ATCC 700392 (strain 26695).

Sequence Analysis

PCR products were purified using an QIAquick PCR purification kit (QIAGEN, Valencia, CA, USA) and subjected to sequencing reactions using the ABI PRISM BigDye™ Terminator version 3.0 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) and an ABI-3100 machine (Applied Biosystems). All analyses were performed in duplicate.

Results and Discussion

H. pylori isolates from 50 clarithromycin-untreated patients were tested for susceptibility to clarithromycin using agar antibiotic dilution method. Primary clarithromycin resistance was found in 10 patients (20%), which is in agreement with literature data [17,18]. The other 40 patients were colonized only with clarithromycin-sensitive strains. The overall prevalence of primary clarithromycin resistance in *H. pylori* isolates from the population in the Metropolitan Region of Chile is higher than in VIII Region of the country (2%) [12]. Also, we observed mixed colonization by both mutant and wild-type *H. pylori* variants in the same patient. Coexistence of clarithromycin-resistant and clarithromycin-sensitive strains in the same geographic area and in the same patient, including cases with no history of clarithromycin exposure, has been previously reported [19,20].

In *H. pylori*, clarithromycin resistance has been associated with a number of different 23S rRNA gene mutations. To determine the frequencies of two common clarithromycin resistance-associated mutations, A2142G and A2143G, PCR products obtained from the clarithromycin-resistant isolates were analyzed by PCR-RFLP (Table 1). No additional *Mbo*II-, *Bbs*I- or *Bsa*I-digestion fragments were detected in the amplified products obtained from all

Table 1 Restriction fragment length polymorphism analysis of the 1402 bp 23S rRNA PCR fragments from 10 primary *H. pylori* clarithromycin resistants

Cases	*Mbo*II	*Bbs*I	*Bsa*I
9/10	+	+	−
1/10	−	−	+
0	+	−	−
0	−	+	−

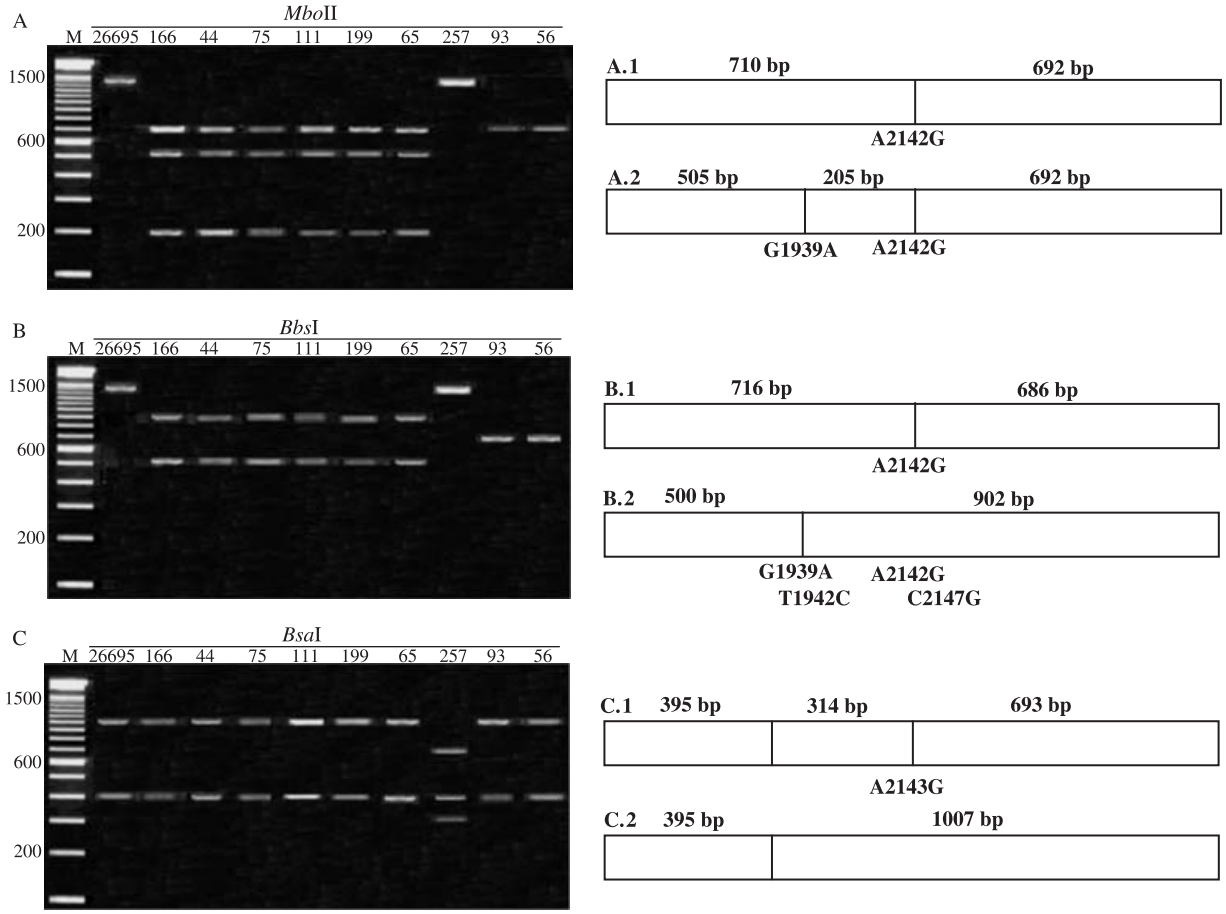

Figure 1 Detection of the 23S rDNA A2142G and A2143G substitutions by *Mbo*II-, *Bbs*I-, and *Bsa*I-mediated polymerase chain reaction-based restriction fragment length polymorphism (PCR-RFLP). Substitutions A2142G and A2143G create restriction sites into the 23S rDNA 1402 bp PCR fragment (panels A1, B1, C1). The 23S rDNA fragment derived from the clarithromycin-sensitive reference ATCC 700392 (strain 26695) has no restriction site for either *Mbo*II or *Bbs*I but it is cleaved into two fragments (395 bp and 1007 bp) by *Bsa*I. Digestion of the PCR fragments of the Chilean clarithromycin-resistant isolates, except isolate 257, produce the same restriction pattern as *Bsa*I-digested DNA fragment from strain 26695. M, 100-bp DNA step ladder (Invitrogen). Panels A1, B1, and C1, restriction patterns of clarithromycin-resistant isolates as described in the literature. Panels A2, B2, and C2, restriction patterns observed in the Chilean clarithromycin-resistant isolates.

the 40 clarithromycin-sensitive strains (not shown). This observation was confirmed by nucleotide sequencing the PCR-amplified fragment from five random clarithromycin-sensitive strains. All those sequences were identical to that of the clarithromycin-sensitive control strain 26695.

Nucleotide sequencing confirmed that nine of the *Mbo*II-positive and nine of the *Bbs*I-positive isolates carried the A2142G mutation (Table 1 and Fig. 1A2). Six of these mutants presented a novel G→A substitution at nucleotide position 1939 (G1939A), thus exhibiting a new restriction site for the *Mbo*II endonuclease (Fig. 1A). Also, nucleotide sequencing showed a novel T→C substitution at nucleotide position 1942, which resulted in a new restriction site for *Bbs*I (Fig. 1B,1B2), and a novel mutation, C2147G, that abrogated the restriction site for the *Bbs*I endonuclease between positions 2142 and 2147 (Fig. 1B,1B2). This does not allow the assignment of a functional role to G1939A, T1942C, and C2147G. However, considering that these novel mutations affect domain V and that region has a

Table 2 Distribution of 10 primary *H. pylori* clarithromycin-resistant isolates according to sequence analysis

Cases	Mutations in the peptidyl transferase region
3/10	A2142G
1/10	A2143G
0	A2142G/A2143G
0	G1939A
0	T1942C
0	C2147G
6/10	G1939A/T1942C/A2142G/C2147G

Table 3 Distribution of minimum inhibitory concentrations among *H. pylori* clarithromycin-resistant isolates. R (as in 1.0R, 1.25R, etc.) refers to maximum concentration of clarithromycin that the *H. pylori* isolate can tolerate; S (as in 1. 25S, 1.5S, etc.) refers to minimum concentration of clarithromycin that will kill at least 99% of the culture

Clarithromycin phenotype (μg/mL)	1.0R-1.25S	1.25R-1.5S	1.5R-1.75S	1.75R-2.0S
No. of isolates	1	3	1	5

strong effect on the secondary structure of the 23S rRNA [21], it is conceivable that those mutations might contribute to antibiotic resistance.

The A2143G substitution, which is *Bsa*I-positive (Table 1), was present only in one of the isolates (Fig. 1C). However, that variant did not carry any of the new described mutations (G1939A, T1942C, and C2147G). Of 10 cases, the A2142G mutation was observed in nine and the A2143G mutation in one (Table 2). According to nucleotide sequencing, both substitution mutations were not present simultaneously. The nine *Bsa*I-negative cases carried only the A2142G substitution and lacked the A2143G substitution that abrogated the *Bsa*I restriction site (Fig. 1C,1C2). Altogether, these results make advisable the use of more than one restriction enzyme to confirm the PCR-RFLP analysis in the characterization of clarithromycin resistance in *H. pylori*. For instance, the clarithromycin-resistant sample 257 showed a restriction pattern corresponding to clarithromycin-sensitive strains when analyzed by PCR-RFLP using *Mbo*II or *Bbs*I due to the absence of the A2142G mutation as defined by DNA sequencing (Fig. 1A,1B). Identical apparent discordances were observed when the other nine clarithromycin-resistant strains were subjected to restriction analysis using *Bsa*I (Fig. 1C).

It has been previously reported that mutations in the *H. pylori* 23S rRNA gene at position 2142 confer a higher level of clarithromycin resistance (≥ 8 μg/mL) than mutations at position 2143 (≤ 4 μg/mL) [22]. None of the Chilean isolates showed a high level of clarithromycin resistance since the MIC of clarithromycin for these 10 isolates, according to the agar antibiotic dilution method, ranged between 1.0 and 2.0 μg/mL (Table 3).

In conclusion, we detected novel variants of the *H. pylori* 23S rRNA gene that affect domain V. Mutations were observed only in clarithromycin-resistant strains and probably they might also contribute to clarithromycin resistance.

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