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Phenotypic and Genotypic Analysis of Clarithromycin-Resistant *Helicobacter pylori* from Bogotá D.C., Colombia

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Resistance of *Helicobacter pylori* to clarithromycin is the most common cause of treatment failure in patients with *H. pylori* infections. This study describes the MICs and the presence of 23S rRNA mutations of *H. pylori* isolates from Bogotá, D.C., Colombia. *H. pylori* were isolated from gastric biopsies from patients with functional dyspepsia. Clarithromycin susceptibility was investigated by agar dilution and strains were considered resistant if the MIC was ≥1 μg/ml. DNA sequences of the 23S rRNA gene of strains resistant and sensitive to clarithromycin were determined to identify specific point mutations. Clarithromycin resistance was present in 13.6% of patients by agar dilution. The A2143G, A2142G and A2142C mutations were found in 90.5, 7.1, and 2.4% of *H. pylori* strains with resistance genotype.The resistant phenotype was associated with 23S rRNA resistance genotype in 85.7% of isolates. The point mutations in 23S rRNA were well correlated with MICs values for clarithromycin.

Keywords: *Helicobacter pylori*, clarithromycin resistance, 23S rRNA mutations

Introduction

Helicobacter pylori, is a microaerophilic Gram-negative bacteria associated with gastroduodenal disorders including peptic ulcer disease and gastric cancer (Graham *et al.*, 1991; Yamaoka *et al.*, 1999; Kato *et al.*, 2000; Malfertheiner *et al.*, 2007; Vakil and Megraud, 2007; Graham *et al.*, 2008; Rimbara *et al.*, 2011). Clarithromycin is one of the most common drugs recommended as first-line eradication therapy for *H. pylori* infection, and has been often used in combination with a proton pump inhibitor and amoxicillin (Hirata *et al.*, 2010). Clarithromycin resistance is regarded as the key factor involved in *H. pylori* therapy failure (De Francesco *et al.*, 2010b). Treatment success varies among regions consistent with the variations in the pattern of resistance. The frequency of *H. pylori* isolates resistant to clarithromycin varies geographically; such as 49.2% in Spain, 40.7% in Japan and moderate frequencies in most of Europe, Asia, and North America (10–20%) (De Francesco *et al.*, 2010a). In Colombia the prevalence of clarithromycin resistance has also been report to vary by region being lower in the "Viejo Caldas" (Manizales, Pereira, and Armenia) (ranging from 2.2–3.8%) (Álvarez *et al.*, 2009a, 2009b) and with moderate frequencies in Bogotá (15–17.7%) (Henao *et al.*, 2009; Trespalacios *et al.*, 2010).

Clarithromycin binds to bacterial peptidyltransferase (E.C. 2.3.2.12); an enzyme associated with the 23S rRNA present in the 50S ribosomal subunit, where it inhibits the elongation step at the translation process. Resistance to clarithromycin results from mutations in the 23S rRNA genes preventing binding. The most prevalent mutations in the 23S rRNA gene occur in two adjacent positions; 2142, and 2143, and include an adenine-guanine transition at position 2142 (A2142G) or 2143 (A2143G) and an adenine-cytosine transversion at position 2142 (A2142C) (Barile *et al.*, 2010). Point mutations observed in low-level clarithromycin resistant *H. pylori* isolates include A2144G and C2694A (Fontana *et al.*, 2002; Rimbara *et al.*, 2008, 2011). T2717C, A2115G, A2116G, G2141A, A2142T, and T2182C are other mutations located in 23S rRNA gene that have been associated with clarithromycin resistance (Taylor *et al.*, 1997; Versalovic *et al.*, 1997).

There have been a few studies in Colombia regarding the prevalence of clarithromycin resistance (Alarcón *et al.*, 2002; Vallejos *et al.*, 2007; Álvarez *et al.*, 2009a; Henao *et al.*, 2009); only one described the A2143G and A2142G mutations as responsible of the clarithromycin resistance in Colombia (Álvarez *et al.*, 2009b).

In the present study the clarithromycin resistance by agar dilution and the presence of point mutations at 23S rRNA by direct sequence in *H. pylori* strains were compared using strains obtained in Bogotá-Colombia between 2009 and 2011.

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Materials and Methods

Ethics and patient's inclusion criteria

Gastric biopsy samples from adult patients referred for gastroscopy at the Gastroenterology Unit of Clínica Fundadores, Bogotá, Colombia; from January 2009 to April 2011 were included in the study. Written informed consent for participation was obtained from each of the patients before entry into the study. Ethical committee of Pontificia Universidad Javeriana and Clínica Fundadores approved the protocol.

Were excluded patients with severe concomitant diseases such as chronic renal failure, decompensate heart failure, HIV, respiratory failure, tumors or malignant diseases, previous gastric surgery, as well as anticoagulated patients, patients addicted to recreational drugs or alcohol, pregnant or lactating women, and women in reproductive age using no family planning method. Patients under antimicrobial therapy during the last three month before entering the study or treatment with antisecretory or inhibitors of the proton pump during the last one month before entering the study were also excluded.

Clinical isolates

Gastric biopsies were preserved at 2 to 8°C in 500 ml of Brucella broth (BD) supplemented with 20% (v/v) glycerol (Invitrogen, USA) for a maximum of 24 h until processing (Ansorg *et al.*, 1991). Biopsies were macerated in aseptic and sterile conditions with a wooden applicator, previously treated with activated charcoal solution (1%, w/v) until total tissue homogenization was achieved (Correa *et al.*, 2000). Subsequently, the homogenate was plated on Wilkins Chalgren agar (Invitrogen) modified for *H. pylori*, by enrichment with IsoVitaleX (BD) and Dent antibiotic supplement (Oxoid), and incubated at 37°C in microaerophilic conditions [10% (v/v) CO_2] for 3 to 14 days. (Arévalo-Galvis *et al.*, 2012).

Identification of *H. pylori*

After incubation, small transparent colonies were selected and tested by Gram stain (Gram-negative curved rods), oxidase (positive), catalase (positive), and rapid urease tests (positive) for confirmation (Ansorg *et al.*, 1991). Colonies which corresponded with the expected profile were isolated in modified Wilkins Chalgren agar (without antibiotics, Invitrogen) and incubated at 37°C in microaerophilic conditions [10% (v/v) CO_2] for 3 to 5 days to obtain pure cultures. If more than one isolate per patient were obtained, they were studied separately. Three replica of each isolate were stored in Brucella broth with glycerol 10% (v/v) at -70°C.

Clarithromycin susceptibility testing

To evaluate the susceptibility to clarithromycin, the minimum inhibitory concentration (MIC) were tested by agar dilution according to the recommendations of Clinical and Laboratory Standards Institute (M100-S20) using Mueller Hinton agar (BD) supplemented with 5% (v/v) sheep or horse blood (aged 2 weeks). Petri dishes containing antibiotic concentrations from 0.125 to 64 mg/ml were prepared and inoculated with 3 ml of *H. pylori* cell suspension (MacFarland scale tube # 2). *H. pylori* NCTC 11637 was used as a control for each susceptibility test. The breakpoint used to determine resistance to clarithromycin was ≥1 μg/ml. The MIC of each isolate was interpreted as the lowest concentration of antibiotic that inhibited the visible growth of *H. pylori* (CLSI, 2010).

H. pylori DNA extraction

H. pylori isolates were cultured in liquid media following the Duque-Jamaica *et al.* (2010) protocol and after 12 h of incubation 2 ml of liquid culture were centrifuged at 10,000×g for 4 min. The supernatant was discarded and DNA extraction was performed using the DNAzol® Kit (Invitrogen).

Detection of mutations at the 23S rRNA gene

Based on 23S rRNA gene sequence of *H. pylori* (GenBank accession no. U27270), two pairs of primers were used to amplify two fragments of the gene encoding the peptidyltransferase. The first set of primers K1 (5′-CCA CAG CGA TGT GGT CTC AG-3′), are complementary to the region ranging from 2,191 to 2,210 bp and K2 (5′-CTC CAT AAG AGC CAA AGC CC-3′), complementary sequences to the region ranging from 2,596 to 2,615 bp were used to amplify a 425 bp fragment (this fragment has been reported to contain mutations at positions 2142, 2143, 2144 (Kim *et al.*, 2002). A second set of primers K3 (5′-GCA CAA GCC AGC CTG ACT G-3′), sequences complementary to the region ranging from 2,786 to 2,804 bp and K4 (5′-AGC AGT TAT CAC ATC CGT G-3′) sequences complementary to the region from 3,181 to 3,199 were used to amplify a 414 bp fragment (in this fragment have been found mutations at positions ranging from 2,694 to 2,717 bp) (Kim *et al.*, 2002).

PCR amplification of DNA was carried out using PCR Master Mix kit (Promega, USA), adding 5 μl of DNA (100 ng), 1 pmol/L of each primer (K1 and K2) or (K3 and K4) on a 50 μl final reaction volume. Amplification conditions were: a denaturation step for 3 min at 94°C, followed by 35 cycles (30 sec for denaturation at 94°C, 30 sec for annealing at 54°C, 30 sec for extension at 72°C) followed by a final extension step at 72°C during 5 min. The amplification products (425 and 414 bp) were resolved on agarose gels 1.5% (w/v), stained with SYBR Safe (Invitrogen) for 1 h. PCR products were purified and sequenced in Macrogen, Korea. Sequences were compared and analyzed using the tool BLASTN (Basic Local Alignment Search Tool); as control the sequence U27270 reported in GenBank was used. The number of nucleotide positions in sequences was established using the Taylor *et al.* (1997) system. Statistic analysis with X^2 test was conducted for assessment of relationship between MIC to clarithromycin by agar dilution and point mutations in 23S rRNA gene.

Results

Clarithromycin resistance prevalence

256 patients infected with *H. pylori* (ages between 19 and

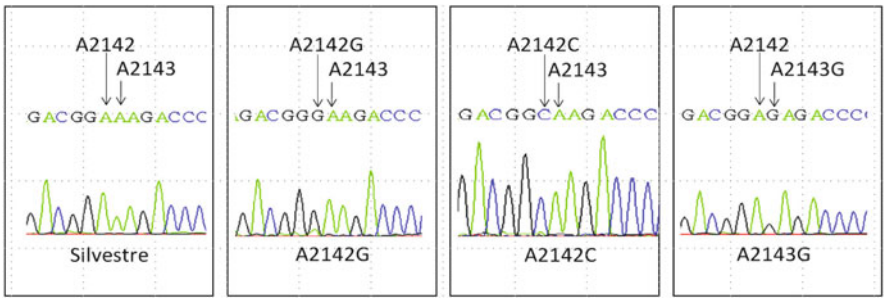

Fig. 1. Results of the sequence of the PCR products amplified with primers K1 and K2. The mutated sequences show the nucleotide change at position 2,142 or 2,143. Double mutation sequences were not observed. (A) Sequence of wild type strain without mutations at positions 2,142 and 2,143. (B) Sequence with the mutation A2142G. (C) Sequence with the A2142C mutation. (D) Sequence with the mutation A2143G.

70 years) were included in the study. Clarithromycin susceptibility by agar dilution showed that 35/256 patients (13.6%) were infected with *H. pylori* resistant strains. Mixed infection (resistant isolates combined with a susceptible strains) was found in 4/256 patients (1.6%).

276 isolates (two or more strains from some pacients) were obtained from 256 patients enrolled in the study. Analyses of MIC and mutations were performed for the total number of strains isolated.

Phenotypic clarithromycin resistance

The MIC for the all-276 strains was determined by the agar dilution method; 42 isolates were resistance to clarithromycin. The range of susceptible strains MIC was 0.016 to 0.5 μg/ml and the clarithromycin resistant isolates MIC ranged between 1 to 32 μg/ml.

Genotypic clarithromycin resistance

DNA from the 42 isolates contained mutations in the fragment amplified with primers K1 and K2 at positions 2,142 and 2,143. No mutations were found using the primers K3 and K4. The distribution of the point mutations among 42 *H. pylori* strains were at A2143G in 38 cases (90.5%), A2142G in 3 cases (7.1%), and A2142C in 1 case (2.4%) (Fig. 1).

Correlation between agar dilution and sequence analysis

The mutation A2142G showed high level resistance to clarithromycin (MIC≥16 μg/ml); while MICs of the A2143G mutants ranged from 1 to 8 μg/ml. There was a strong association between the presence of 23S rRNA gene mutations and clarithromycin resistance. Overall, 36 of 42 (85.7%) clarithromycin resistant strains by agar dilution contained a point mutations in their 23S rRNA sequences. There was discrepancy between both methods for 12 strains. 6 (14.2%) *H. pylori* strains were resistant to clarithromycin by agar dilution but we did not find point mutations in 23S rRNA. Additionally; 6 (14.2%) sensitive strains by agar dilution had point mutations in 23S rRNA gene (Figs. 1 and 2). Statistical analysis showed that significant differences between the proportion of resistant strains with presence of mutation and the proportion of susceptible isolates without mutation, $P<0.0001$.

Discussion

The frequency of resistance to clarithromycin found (13.6%) was slightly lower but similar our previous experience reported for the period 2008 to 2009 (17.7%) in Bogotá (Trespalacios *et al.*, 2010), and also similar to that reported

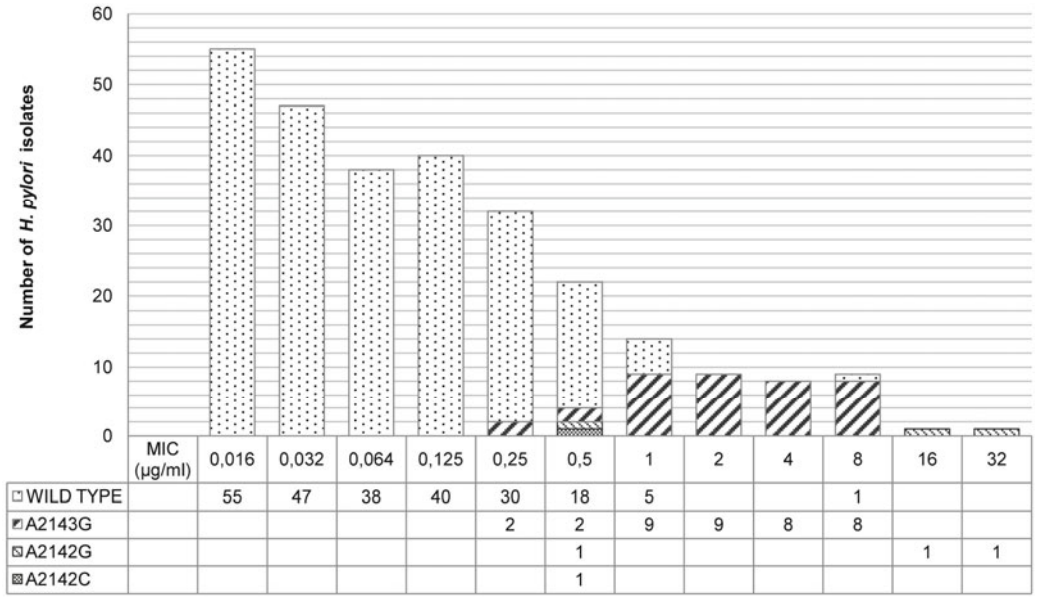

Fig. 2. Distribution of *H. pylori* isolates according MICs and 23S rRNA mutations.

by Henao *et al.* (2009) in isolates from 2007 (15%) who were processed between within 24 h of have been collected in Bogotá. Slight differences in the prevalence of resistance to clarithromycin could respond chance, differences in techniques used to determine the susceptibility, or both. In the Henao *et al.* (2009) study the susceptibility was determined by disk diffusion. In our first study (Trespalacios *et al.*, 2010) susceptibility was determined by E-test. In the present study MIC was determined by agar dilution.

To identify mutations associated with resistance to clarithromycin we amplified two regions of 23S rRNA. The mutation A2143G was the most frequent (90.5%), followed by A2142G (7.1%) and A2142C (2.4%). In USA between 48% to 53% of resistant isolates carry the mutation A2142G (Stone *et al.*, 1997; Versalovic *et al.*, 1997); in Europe the reported frequencies for A2142G range from 23% to 33% for A2142G, 44 to 67% for A2143G and 2 to 10% for A2142C (Alarcón *et al.*, 2000; van Doorn *et al.*, 2001). In Spain the most frequent mutation in 23S rRNA is A2143G (85%) (Agudo *et al.*, 2010). Similar results have been observed in Japan and China, where more than 90% of the strains carry the mutation A2143G (Maeda *et al.*, 2000; Kato *et al.*, 2002; Pan *et al.*, 2002). Our findings are consistent with the previous work done in southern Colombia where the mutation A2143G was the most frequent (82%) (Álvarez *et al.*, 2009b).

We founded discrepancy between phenotypic and genotypic methods in 12 strains, 6 of them strains harbouring genotypic resistance that does not emerge phenotypically; these difference between the two methods is mainly due to the detection of a heteroresistant status by sequence method; understanding heteroresitant status as the co-existence of strains susceptible and resistant to the same pacient (De Francesco *et al.*, 2010c), Additionally; two copies of the 23S rRNA gene exist in *H. pylori* chromosomal DNA, and a mutation in one copy has been shown to be enough to confer clarithromycin resistance. Indeed, the mutations may be heterozygous, and the sequence method could be detect the mutation. However, because of the apparent rarity of heterozygosity, the mix infection or heteroresistant status is most likely (Marais *et al.*, 1999).

In cases of phenotypic resistance with genotypic susceptibility, that discordant results may occur due the presence of point mutations not investigated or other genetic mechanisms, such as efflux pumps or RNA methylation (Liu *et al.*, 2008; De Francesco *et al.*, 2010c; Hirata *et al.*, 2010). Our study also confirmed a strong association between MICs and point mutations in 23S rRNA gene. The high degree of association between clarithromycin resistance in *H. pylori* Colombian strains and point mutations in the peptidyltransferase of 23S rRNA gene suggests that molecular testing will be usefully clinically in Colombia and enable susceptibility testing without need for culture from biopsy samples.

Conclusion

We identified the clarithromycin susceptibility phenotypes (MICs) in *H. pylori* isolates from Bogotá D.C., Colombia. MIC ranged between 0.016 to 32 μg/ml. The most common mechanism associated with resistance to clarithromycin was the A2143G mutation at 23S rRNA gene which is consistent with the previous results obtained in southern Colombia. These studies suggest that molecular method will be useful for identify resistant strains. Our results also confirmed the good correlation between the results of sequence analysis and agar dilution. In conclusion, the prevalence of *H. pylori* clarithromycin resistance was of 13.6% in Bogotá, Colombia. Molecular testing for clarithromycin susceptibility is a good alternative for determining the whether to use clarithromycin clinically and is especially useful in Colombia, a country where culture and sensitivity test for *H. pylori* is not routinely available.

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