



ORIGINAL ARTICLE

Usefulness of rapid urease test samples for molecular analysis of clarithromycin resistance in *Helicobacter pylori*

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Abstract *Helicobacter pylori* is a gastric pathogen that is widely recognized as a causative agent of gastric disease. Its eradication is variable, mainly due to increased resistance to clarithromycin. Our objective was: to evaluate (i) if the biopsy specimen used for the rapid urease test is a useful sample to detect resistance to clarithromycin by PCR-RFLP and (ii) the distribution of A2142G and A2143G point mutations in the 23S rRNA gene, in relation to virulence factors in our region. Gastric specimens were collected from adult dyspeptic patients (n = 141) and *H. pylori* was investigated by the rapid urease test, histopathological analysis and PCR for the *hsp60* gene. Clarithromycin resistance was detected by PCR-RFLP in 62 *H. pylori* (+) paired biopsy specimens submitted to molecular analysis and the rapid urease test. *H. pylori* virulence factors were analyzed by multiplex PCR using specific primers for the *cagA*, *vacA* and *babA2* genes. Thirteen out of 62 strains (20.9%) were resistant to clarithromycin: 6/13 (46.2%) harbored the A2143G mutation whereas 7/13 (53.8%) carried the A2142G point mutation. *vacA* m1s1 was the most frequent genotype among the resistant strains. In conclusion, the biopsy specimens used for the rapid urease test were suitable samples for clarithromycin resistance detection in patients infected with *H. pylori*, which became especially useful in cases where the number or size of the biopsies is limited. In addition, this is the first report of a molecular analysis for clarithromycin resistance performed directly from gastric biopsies in our region.

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PALABRAS CLAVE

Helicobacter pylori;
Resistencia a
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Factores de
virulencia;
PCR-RFLP;
Gastritis;
Cáncer gástrico

Utilidad de las muestras de test rápido de ureasa para el análisis molecular de resistencia a claritromicina en *Helicobacter pylori*

Resumen *Helicobacter pylori* es un patógeno ampliamente reconocido como causante de enfermedad gástrica. Su erradicación es variable, principalmente debido al incremento de la resistencia a claritromicina. Nuestros objetivos fueron evaluar la utilidad de la biopsia usada para realizar el test rápido de ureasa en la detección de resistencia a claritromicina por PCR-RFLP y conocer la distribución de las mutaciones puntuales A2142G y A2143G en el gen ARNr 23S, en relación con los factores de virulencia en nuestra región. Se recolectaron muestras gástricas (n=141) provenientes de pacientes adultos dispépticos y se investigó la presencia de *H. pylori* mediante el test rápido de ureasa, análisis histopatológico y PCR para el gen *hsp60*. La resistencia a claritromicina se analizó por PCR-RFLP en 62 muestras pareadas de biopsias gástricas *H. pylori*+ destinadas al análisis molecular y al test rápido de ureasa. Los factores de virulencia de *H. pylori* fueron analizados mediante PCR multiplex usando oligonucleótidos específicos para los genes *cagA*, *vacA* y *babA2*. Trece de 62 cepas (20,9%) fueron resistentes a claritromicina, 6/13 (46,2%) llevaron la mutación A2143G, mientras que 7/13 (53,8%) presentaron la mutación A2142G. El genotipo *vacA* s1m1 fue el más frecuente entre las cepas resistentes a claritromicina. En conclusión, las biopsias destinadas al test rápido de ureasa fueron muestras apropiadas para la detección de la resistencia a claritromicina en pacientes infectados con *H. pylori*. Esto es especialmente útil en aquellos casos en los que el número o el tamaño de las muestras son limitados. Además, este es el primer reporte de estudio de resistencia a claritromicina (mediante técnicas moleculares), directamente de biopsias gástricas en nuestra región.

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Introduction

Helicobacter pylori is an etiological agent for several gastroduodenal diseases with various clinical manifestations ranging from dyspepsia, chronic gastritis, gastric atrophy and peptic ulcer disease to gastric adenocarcinoma and primary gastric B-cell lymphoma. The different clinical presentations may be due to specific virulence factors of the bacterium (among others, *cagA*, *vacA* and *babA* genes) as well as intrinsic host factors. Furthermore, it has been classified as a Class I carcinogen by the International Agency for Research on Cancer (IARC)²⁷ as well as by the World Health Organization. *H. pylori* is known to colonize the gastric mucosa in 50% of the human population worldwide, with varying prevalence rates among different geographical regions, with the highest rates observed in developing countries^{13,27}. In this regard, the detection of *H. pylori* in gastric biopsies includes bacterial culture, the rapid urease test and molecular analysis by nucleic acid amplification.

Early eradication of *H. pylori* has been proven to reduce the incidence rate of gastric cancer and improve ulcer healing²⁷. Nowadays, the most widely used treatment regimens include the standard triple therapy, which comprises two out of three antibiotics, clarithromycin and amoxicillin or metronidazole, together with a proton pump inhibitor (PPI)¹⁶. The efficacy of this therapy has dramatically declined over the last decade, mainly due to increasing clarithromycin resistance rates observed in many regions of the world²¹. In Argentina there are few reports about clarithromycin resistance; however, current evidence indicates that such resistance is increasing^{24,29–31}. Furthermore, as reported by Zerbetto de Palma et al.³¹,

such clarithromycin-resistance rate increase is tied to an increased rate in multidrug resistance.

Clarithromycin activity depends on the capacity to inhibit protein synthesis by binding to the bacterial 50S ribosomal subunit and point mutations in the peptidyl transferase region encoded in the domain V of 23S rRNA are responsible for macrolide resistance by inhibition of the binding between the antibiotic and the ribosomal unit. The most frequent mutations associated with clarithromycin resistance are A2143G and A2142G in the 23S rRNA gene and, to a lesser extent, A2142C²⁶.

Phenotypic and genotypic resistance to clarithromycin can be assessed by culture (agar diffusion, agar dilution, broth microdilution, E-test) and PCR-based techniques, respectively. Because of the slow growth and particular requirements of *H. pylori* culture, phenotypic antimicrobial susceptibility tests are not routinely performed in clinical practice. For this reason, molecular tests targeting *H. pylori* resistance-associated gene mutations appear as a useful alternative for the detection of clarithromycin resistance. Taking into account the above mentioned data, the aims of this study were to evaluate: (i) if the biopsy samples used for the rapid urease test is a useful sample to detect resistance to clarithromycin by PCR-RFLP and (ii) the distribution of A2142G and A2143G point mutations in relation to virulence factors in our region.

Materials and methods

Adult dyspeptic patients (n = 141) attending the Gastroenterology Service of "Dr. José María Cullen" Hospital (Santa Fe, Argentina) from February 2014 to March 2015 were

enrolled in the study, after informed consent was obtained. All of them underwent upper gastrointestinal endoscopy. Patients who had received antibiotic therapy and/or PPIs in the last 15 days were excluded from the study.

H. pylori detection: gastric specimens were collected according to the modified Sydney Protocol. *H. pylori* was investigated by the rapid urease test, histopathological analysis and PCR for the *hsp60* gene.

DNA from patients whose samples were *H. pylori*-positive by the histopathological test and PCR amplification of the *hsp60* gene¹² were included for resistance testing and for virulence factors analysis. DNA extraction was carried out as follows: biopsy samples for the rapid urease test as well as the molecular analysis were collected in Christensen's urea broth and in saline solution, respectively; in the molecular diagnostics laboratory, solutions were discarded; 400 µl TES and 2 µl of 20 mg/ml proteinase K were added and tubes were incubated for 2 h at 60 °C (or overnight at 4 °C). Then, 125 µl of 5 M NaCl were added. After mixing, 400 µl of chloroform were added and tubes were vigorously shaken until a milky solution formed. Tubes were centrifuged at 10 000 × g for 3 min and the upper aqueous phase was transferred to a new tube (600 µl). Equal volume (600 µl) of cold 95% ethanol (−20 °C) was added and tubes were inverted 3 times to condense the DNA. A centrifugation at 10 000 × g for 2 min was carried out and the supernatant was removed. The pellet was washed with 1 ml cold 70% ethanol (−20 °C) and each tube was centrifuged for 1 min at 10 000 × g. After discarding the supernatant, the DNA was dried by placing the tube upside down on absorbent paper. Finally, the dried pellet was resuspended in 50–200 µl of TE 10:1 pH 8.0 (with vortex) and incubated at 56 °C until complete dissolution.

PCR-RFLP (clarithromycin resistance detection). PCR was performed using the primers described by Suzuki et al.²⁶ which amplified a fragment of 768 pb corresponding to the domain V of the 23S rRNA gene: Hp23Sr6 sense (5' - CACACAGGTAGATGAGATGAGTA-3') and Hp23Sr7 antisense (5' - CACACAGAACCAACCGGATCACTA-3'). An individual PCR reaction (final volume: 25 µl) was done by a mix consisting of 12.5 µl of 2X GoTaq Green MMix (Promega, USA), 1 µl of forward/reverse primers mix (10 µM), 2.5 µl of DNA and 9 µl nuclease-free water. PCR conditions were: 94 °C 5 min followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s and extension at 72 °C for 30 s and one cycle at 72 °C during 7 min. Two aliquots of the amplification reactions (10 µl) were digested separately with *MbolI* and *BsaI* (New England BioLabs) for 1 h at 37 °C. These enzymes identified mutations in the *H. pylori* domain V of the 23S rRNA gene at positions 2142 and 2143, respectively.

Virulence factor detection. The *cagA*, *babA2* and *vacA* genes were analyzed by multiplex PCR using sequence specific primers (0.4 µM each one) and GoTaq® Green Master Mix, (Promega Corp) (25 µl/tube)^{8,10,12}. PCR cycling conditions were: 94 °C for 5 min followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s and extension at 72 °C for 30 s. Finally, a cycle at 72 °C for 7 min was carried out. In all cases, PCR products were visualized after electrophoresis in 2% agarose gel stained with Sybr Safe (Life Technologies, USA).

Results

Biopsies of 62 adult patients infected with *H. pylori* (as assessed by PCR and histology) were analyzed by PCR-RFLP for the clarithromycin-resistance analysis. The test was performed simultaneously on biopsy samples destined to molecular analysis as well as those for the rapid urease test. In both samples, a 768-bp product can be specifically amplified with similar yield. In order to investigate the presence of A2142G and A2143G mutations, the amplified products were digested with *MbolI* and *BsaI* restriction enzymes. Figure 1 shows wild type samples digested with *MbolI* and *BsaI*, respectively: since no recognition site for *MbolI* was present, amplicons appear unmodified (Fig. 1A); moreover, the 768-bp fragment naturally presents a target sequence for *BsaI* which generates two restriction fragments (108 and 660 bp) (Fig. 1B). Strains carrying the A2142G and A2143G mutations are shown in Figure 2. In the presence of the A2142G mutation the resulting restriction DNA fragments were of 418 bp and 350 bp (Fig. 2A) whereas in the presence of the A2143G mutation the resulting fragments were of 108, 310 and 350 bp (Fig. 2B). No differences in restriction patterns were found between both types of samples. Hence, we used the rapid urease test biopsies to study the prevalence of mutations causing clarithromycin resistance in our region. Thirteen out of 62 strains (20.9%) were resistant to clarithromycin: 7/13 (53.8%) harbored the A2142G mutation whereas 6/13 (46.2%) carried the A2143G point mutation. There were no strains harboring a double mutation. In addition, a multiplex PCR amplification for *cagA*, *vacA* and *babA2* virulence factors was performed on these mutant strains. Figure 3 is a representative image of such assay showing different genotypes of *H. pylori*. Table 1 shows the distribution

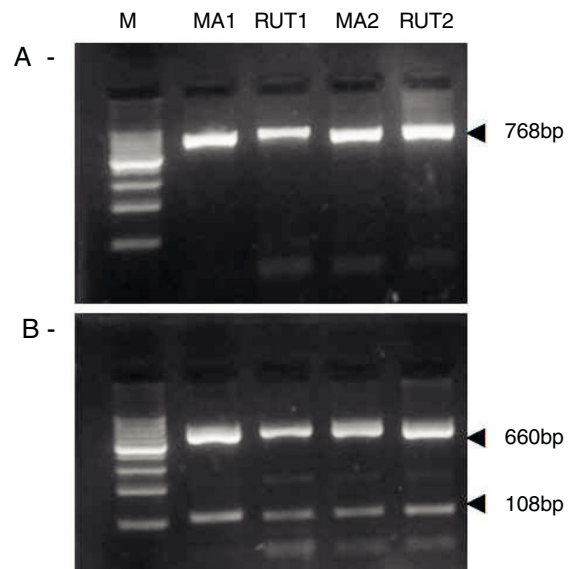


Figure 1 *Helicobacter pylori* clarithromycin resistance analysis by PCR-RFLP. DNA was purified from biopsies for the rapid urease test (RUT) and for molecular analysis (MA). Amplicons (768 bp) from wild type strains were digested with *MbolI* (A) and *BsaI* (B). M, molecular size marker (Cien Marker, Biodynamics, Buenos Aires, Argentina).

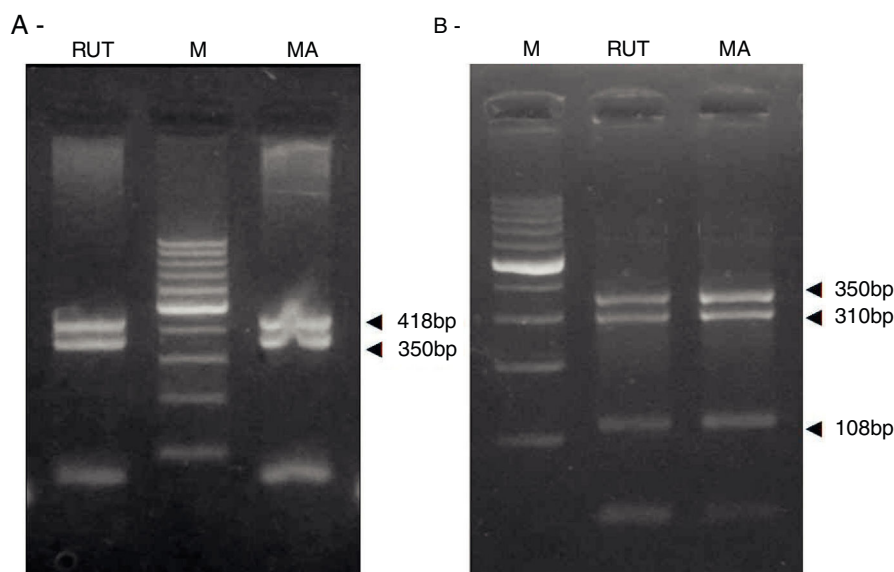


Figure 2 *Helicobacter pylori* clarithromycin resistance analysis by PCR-RFLP. Amplicons (768 bp) from mutant strains were digested with *MbolI* (A) and *BsaI* (B). M, molecular size marker (Cien Marker, Biodynamics, Buenos Aires, Argentina).

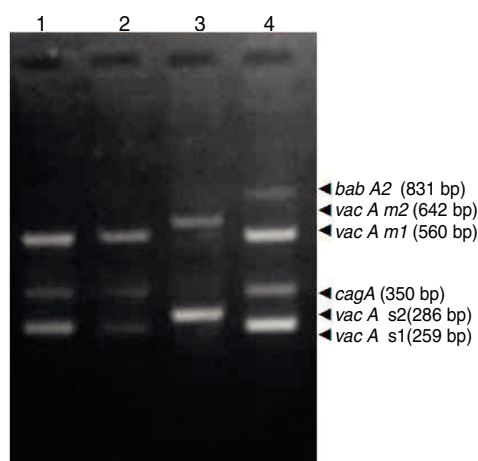


Figure 3 Multiplex PCR for *Helicobacter pylori* virulence factors. Lanes: (1) *cagA*(+)/*vacAs1m1/babA2*(-) genotype, (2) *cagA*(+)/*vacAs1m1/babA2* (-) genotype, (3) *cagA*(-)/*vacAs2m2/babA2*(-) genotype, (4) *cagA* (+)/*vacAs1m1/babA2*(+) genotype.

of the different virulence factors found in samples carrying point mutations responsible for clarithromycin resistance. It can be observed that *vacA* m1s1 was the most frequent genotype among the resistant strains (8/13).

Discussion

The prevalence of clarithromycin resistance in *H. pylori* is increasing worldwide and this is the major cause of treatment failure of *H. pylori* infection. The high consumption of clarithromycin, especially in the treatment of respiratory tract diseases, is probably the main reason of this increased resistance^{19,23,24,29,30}. It is well known that the prevalence of *H. pylori* resistant to clarithromycin varies among the

Table 1 Association between virulence factors and mutation points in the 23S rRNA gene in *Helicobacter pylori*.

UP ^a	<i>cagA</i>	<i>vacA</i>		<i>babA2</i>	Point mutations	
		s	m		A2142G	A2143G
UP01	—	2	2	—	—	+
UP02	—	1	1	—	+	—
UP03	—	2	2	+	—	+
UP04	—	2	1	—	—	+
UP05	—	2	2	—	—	+
UP06	+	1	1	+	+	—
UP07	—	2	1	+	+	—
UP08	—	1	1	—	+	—
UP09	—	1	1	—	—	+
UP10	—	1	1	+	+	—
UP11	—	1	1	+	+	—
UP12	—	1	1	—	—	+
UP13	—	1	1	+	+	—

^a UP: unidentified patient.

different countries. The resistance rate in Japan was around 25.9% in 2014; in Europe, the lowest clarithromycin resistance was reported in Norway (5.9%), while the highest in Spain (32.01%) and Portugal (42.35%). Recently, Ghotaslou et al.¹¹ reported the clarithromycin resistance rates in North America (30.8%) and South America (12.9%). Many Latin American countries exhibit a high rate of *H. pylori* infection and associated diseases with a global clarithromycin resistance prevalence of 13%, with high heterogeneity between different studies⁷. Since the latest Maastricht Guidelines recommended clarithromycin-containing schemas as first-line therapy in regions with low clarithromycin resistance (<15–20%)¹⁶, it is important to know the local susceptibility of *H. pylori* isolates to guide empirical treatment in clinical practice. Although point mutations in target genes are responsible for antibiotic resistance, in routine

clinical microbiology the prevalence of resistance is often determined by phenotypic methods. It is so because new mechanisms of resistance not related to point mutations at antimicrobial target genes can emerge⁴ and, moreover, the prevalence of known mutations in the target genes may shift to novel ones. Furthermore, even if a positive test result of a genotypic method in one biopsy confirms resistance, it must be considered that a negative result does not rule out resistance, due to the fact that it is possible that isolates in different biopsies evolve in an independent fashion.

Nevertheless, due the slow growing properties of *H. pylori*, and the specific culture conditions that are needed, the phenotypic approach is challenging and time-consuming. Hence, the usefulness of the molecular approaches to detect point mutations related to clarithromycin-resistance directly from biopsies such as those presented here is very important mainly for low-income clinical laboratories, avoiding the difficulty of phenotypic methods. Moreover, molecular analyses have the advantage of avoiding the effect of heteroresistance in a single niche that can influence MIC results.

Recently, Megraud²⁰ have highlighted the importance of molecular approaches to identify *H. pylori* antimicrobial resistance, especially for tailored therapies, because when point mutations at 23S-rRNA are detected, other empiric therapies can be employed. Taking this fact into account, continuous surveillance of the prevalent mutations could also be carried out, including their detection by genotypic methods. Such approach has been recently used by Ducournau et al.⁹ In this regard, PCR-based commercial methods are currently available to rapidly detect resistance to clarithromycin, providing accurate and convenient information. However, they are still too expensive for a low-income setting. In this study we decided to evaluate if the biopsy specimens used for the rapid urease test could be a useful samples to detect resistance to clarithromycin by PCR-RFLP. We found that PCR-RFLP performed from biopsy specimens submitted to the rapid urease test was appropriate to detect resistance to clarithromycin and thus, it was used to analyze clarithromycin resistance in patients infected with *H. pylori* in our region. In Argentina there are few reports about clarithromycin resistance, however current evidence indicates that such resistance is increasing^{7,24,29–31}. In accordance with those reports, the results of our study showed mutations responsible for clarithromycin resistance in 20.96% of adult patients. However, it must be considered that this percentage could be increased if point mutations other than those studied in our work are taken into account. Unlike other authors^{1,19,22,26}, we did not find a marked predominance between the two mutations, perhaps due to the small number of samples studied. Our results agree with those by Klesiewicz et al.¹⁵, who reported the same frequencies of point mutations (A2142G/A2143G) among clarithromycin-resistant *H. pylori* strains.

Several authors have focused on the relationship among antibiotic resistance and virulence factors of *H. pylori* with contradictory results. Agudo et al.², in Spain, found that clarithromycin-resistant *H. pylori* strains carried the *vacA* s2/m2 genotype most frequently and were most likely to be *cagA*-negative than susceptible strains. On the contrary, Rasheed et al.²³ found a high proportion of *cagA*-positive and

vacA s1m1 genotypes in *H. pylori* isolates, thus indicating antibiotic resistance.

A negative association between the prevalence of virulent (*cagA*+) *H. pylori* strains and the prevalence of clarithromycin resistance has been reported whereas Boyanova et al.⁵ did not find an association between point mutations (A2142G and/or A2143G) and the *cagA* status or the *vacA* S and M alleles; however a close relationship is observed between A2142G/*vacAi*1 and A2143G/*vacAi*2. In regard to the *babA*2 gene, significant rates of clarithromycin resistance were observed by Karabiber et al.¹⁴ in *babA*2- (and *cagE*-, *iceA*1-, and *vacA* s1c-positive) pediatric patients. On the contrary, Alarcón-Millán et al.³ found no significant differences in genotype distribution and clarithromycin resistance or susceptibility. In addition to this complex scenario, two interesting papers show that the co-existence of diverse genotypes of putative virulence factors in a single host and/or microevolution phenomena must be considered when drawing a correlation with the clinical presentation (and perhaps with resistance patterns)^{17,18}.

In our study, like in other reports, *vacA* m1s1 was the most frequent genotype among the resistant strains^{6,29}. Due to the fact that – among the *vacA* genotypes – the *vacA* m1s1 genotype is the most virulent and that previous reports described higher eradication rates when more virulent strains are present^{5,23,25,28} this could emphasize the need for fast and reliable ways to identify, at molecular level, the strains present in *H. pylori*-infected patients in order to select the best eradication treatment and prevent future complications such as pre-neoplastic lesions or progression to gastric cancer. To our knowledge, this is the first study on the analysis of *H. pylori* clarithromycin resistance (and virulence factor distribution) by molecular methods directly from gastric biopsies in this region of Argentina. In addition, this approach is especially useful in cases where the number or the size of biopsies are limited. Currently, we are devoted to optimizing an allele-specific primer-PCR (ASP-PCR) protocol in order to simplify the detection of clarithromycin resistance in *H. pylori*.

Conflict of interest

The authors declare that they have no conflicts of interest.

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