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Improved allele-specific PCR assays for detection of clarithromycin and fluoroquinolone resistant of *Helicobacter pylori* in gastric biopsies: identification of N87I mutation in GyrA

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ABSTRACT

Molecular testing can rapidly detect *Helicobacter pylori* susceptibility using gastric biopsies. Allele-specific polymerase chain reaction (ASP-PCR) was used to identify *H. pylori* 23S rRNA and *gyrA* mutation using gastric biopsies from Colombian patients and confirmed by PCR and sequencing of the 23S rRNA and gyrA genes. The sensitivity and specificity of ASP-PCR were compared with susceptibilities measured by agar dilution. Samples included gastric biopsies from 107 biopsies with *H. pylori* infections and 20 *H. pylori* negative. The sensitivity and specificity of ASP-PCR for the 23S rRNA gene were both 100%. The sensitivity and specificity of ASP-PCR for the *gyrA* gene, published in 2007 by Nishizawa et al., were 52% and 92.7%, respectively; the lower sensitivity was due to the presence of mutation N87I in our samples, which were not detected by the test. In this study, we designed new primers to detect the mutation N87I in GyrA. The ASP-PCR was performed with the original primers plus the new primers. The molecular test with the new primers improved the sensitivity to 100%. In conclusion, ASP-PCR provides a specific and rapid means of predicting resistance to clarithromycin and levofloxacin in gastric biopsies.

1. Introduction

Helicobacter pylori is an important human pathogen that causes gastroduodenal inflammation and is etiologically associated with duodenal ulcer disease, gastric ulcer disease, gastric adenocarcinoma, and primary B-cell gastric lymphoma (Chisholm et al., 2001; Graham, 2009; Heo and Jeon, 2014). The National Institutes of Health in the United States, the Maastricht Consensus in Europe, and the Canadian Consensus all recommend *H. pylori* eradication for the treatment or prevention of these disorders and reduction of the occurrence of new gastric cancers after endoscopic resection (Fontana et al., 2002; Malfertheiner et al., 2012). The most common method for the eradication of *H. pylori* infections consists of the administration of a proton pump inhibitor and several antimicrobial agents such as amoxicillin, clarithromycin, metronidazole, fluoroquinolone, or tetracycline (Furuta and Graham, 2010). Antimicrobial resistance is now the most important factor determining the outcome of *H. pylori* eradication therapy (Fontana et al., 2002). Resistances to clarithromycin and fluoroquinolones are particularly important, as they cannot be overcome by increasing the dose or duration of therapy (Agudo et al., 2010; Furuta and Graham, 2010; Sugimoto et al., 2014). In both, phenotypic resistance is correlated with clinical and microbiological failure. Mutations leading to resistance have been described for macrolides and fluoroquinolones (Cambau et al., 2009; Papastergiou et al., 2014; Wang et al., 2001). Resistance to clarithromycin results from structural changes in the 23S rRNA molecule caused by mutation of the 23S rRNA gene (Papastergiou et al., 2014; Wang et al., 2001). Most common mutations are A-G transitions at position 2143 (A2143G) and 2142 (A2142G) (Ahmad et al., 2009; Cambau et al., 2009; Garrido and Toledo, 2007; Ho et al., 2010; Kim et al., 2002); these mutations have been confirmed to confer resistance by mutagenesis studies (Taylor et al., 1997; Marais et al., 1999). Other mutations observed in low-level clarithromycin-resistant *H pylori* isolates include A2142C, A2144G (De Francesco et al., 2014; Xiong et al., 2013), and C2694A (Rimbara et al., 2008).

Fluoroquinolone resistance is caused by point mutations in the quinolone resistance-determining region (QRDR) of the *gyrA* gene, which encodes subunit A of DNA gyrase (Miyachi et al., 2006). The amino acid substitutions observed in clinical strains have been primarily reported at position 87 (Asn to Lys) or 91 (Asp to Gly, Asp to Asn, or Asp to Tyr) (Alfizah et al., 2014; Cattoir et al., 2007; Hung et al., 2009; Nishizawa et al., 2007), although resistant strains lacking these mutations have also been described (Wang et al., 1999, 2010).

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In routine clinical practice, the detection of clarithromycin and levofloxacin resistance is based on phenotypic methods such as E-test or agar dilution; however, these methods are time consuming, as they require up to 2 weeks for completion. Molecular methods to detect the point mutations conferring resistance have the potential advantage of providing rapid results. A simple method for detection of antibiotic susceptibility using polymerase chain reaction (PCR) would promote the use of "tailored treatment" in the era of increasing prevalence of antimicrobial resistance (Heo and Jeon (2014). Allele-specific PCR (ASP-PCR) is especially useful to determine single nucleotide polymorphism in DNA samples, and this technique allows the identification of mutations without direct sequencing or digestion with restriction enzymes (Furuta et al., 2007; Nakamura et al., 2007; Nishizawa et al., 2007). This study used ASP-PCR to identify mutations predictive of clarithromycin and fluoroquinolone resistance in DNA from gastric mucosal biopsy samples from Colombian patients infected with *H. pylori*. The concordance, sensitivity, and specificity for the ASP-PCR assay were determined by comparing the results of agar dilution (phenotypic test) for each clinical isolate of *H. pylori* and the molecular detection of the mutant in respective biopsy.

2. Materials and methods

2.1. Clinical samples

Gastric biopsies from 127 patients referred for gastroscopy at the Gastroenterology Unit of Clínica Fundadores, Bogotá, Colombia, were entered, including 107 patients with active *H. pylori* infections and 20 *H. pylori*–negative patients. Written informed consent for participation was obtained from each of the patients before entry into the study. The protocol was approved by ethical committee of Javeriana University and Clínica Fundadores. These patients were part of a clinical trial to assess the efficacy of a triple therapy containing levofloxacin. The status of the infection was confirmed by rapid urease test and histopathology (Giemsa stain). Two antral biopsies specimens were obtained of each patient, and 1 of the tissue specimens was cultured for *H. pylori* and the other was used for DNA extraction.

2.2. Bacterial strains, culture conditions, and determination of susceptibility to clarithromycin and levofloxacin

Biopsies samples were crushed in 0.5 mL of Phosphate buffered saline (PBS) and cultured in Wilkins Chalgren Agar (Becton Dickinson, Heidelberg, Germany) containing 7% horse blood, vancomycin (10 mg/L), and trimethoprim (5 mg/L). The plates were incubated at 37 °C under microaerophilic conditions for up to 14 days, and isolates were identified as *H. pylori* by Gram stain, urease, catalase, and oxidase reactions (Kist, 1991).

Susceptibility to clarithromycin was assessed using dilution method according to the CLSI breakpoints: S ≤ 0.25 μg/mL; I = 0.5 μg/mL; R ≥ 1.0 μg/mL (CLSI, 2010). Breakpoint for levofloxacin was determined as R ≥1.0 μg/mL agrees with EUCAST clinical breakpoints for *H. pylori* (EUCAST, 2011; Hung et al., 2009).

2.3. DNA sequencing of 23S rRNA gene and gyrA gene of H. pylori isolates

Total genome DNA was extracted from *H. pylori* isolates using DNAzol® kits (Invitrogen, Carlsbad, CA, USA. The DNA samples were stored at −20 °C until use. Primers 23S rRNA F (5′-CCA CAGCGATGTGGTCTCAG-3′) corresponding to position 2191 to 2210 and 23S rRNA R (5′-CTCCATAAGAGCCAAAGCCC-3′) corresponding to position 2596 to 2615 were used to amplify a fragment of 425 bp of the peptidyltransferase region of the 23S rRNA in *H. pylori* (GenBank accession number U27270) and described by Kim et al. (2002). PCR amplification of DNA was performed using a MyCycler thermal cycler (Biorad, Foster City, California, USA) in a final volume of 50 μL containing 1 μg of *H. pylori* genomic DNA, 1 μmol/L concentration of primers, and 42 μL of Super Mix (Invitrogen). The cycling program was 1 cycle at 95 °C for 5 min; 35 cycles of 95 °C for 30 s, 54 °C for 30 s, 72 °C for 30 s, and a final extension step at 72 °C for 10 min.

Primers corresponding to regions flanking the 428-bp coding sequence of the QRDR of *gyrA* (codons 38–154) were used for the amplification of the *gyrA* gene. The *gyrA* primer sequences were *gyrA* F (5′-TTTRGCTTATTCMATGAGCGT-3′) and *gyrA* R (5′-GCAGACGGCTTGGTA RAATA-3′). The PCR mixture (50-μL final volume) contained 1 μg of *H. pylori* genomic DNA, Super Mix (Invitrogen), and 0.5 μmol/L (each) primer. PCR was performed under the following conditions: initial denaturation at 94 °C for 4 min, followed by 26 cycles of denaturation at 94 °C for 60 s, annealing at 56 °C for 60 s, and extension at 72 °C for 30 s, with a final extension at 72 °C for 5 min.

PCR products (23S rRNA and *gyrA*) were purified by Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, Wisconsin, USA) and sequenced by Macrogen (Korea, Seoul, Republic of Korea).

2.4. DNA preparation and PCR analysis for gastric biopsy specimens

DNA was isolated from the gastric tissue specimens using QIAmp DNA mini kits (Qiagen, Hilden, Germany). DNA preparations were subjected to PCR for *ureA* and *vacA* genes to confirm the presence of *H. pylori* DNA. PCR for the *ureA* gene was performed using primers: *ureA*-F (5′-AACCGGATGATGTGATGGAT-3′) and *ureA*-R (5′-GGTCTGT CGCCAACATTTTT-3′) reported by Kim et al. (2002). The amplification was conducted using GoTag polymerase (WI, USA) under the following conditions: initial denaturation at 94 °C for 2 min, followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 20 s, and extension at 72 °C for 30 s, with final extension 72 °C for 5 min. PCR for the *vacA* gene was performed using GoTag polymerase (WI, USA) and the primers *vacA*-F (5′-TACAACAAACACACCGCAAAA-3′) and *vacA*-R (5′-TGTAGCGATACCCCCAACAA-3′) reported in 2004 by Ayala et al. The PCR condition for the *vacA* gene amplifications was initial denaturation at 94 °C for 2 min, followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 20 s, and extension at 72 °C for 30 s, with final extension 72 °C for 5 min.

2.5. ASP-PCR to determine 23S rRNA mutation of H. pylori from DNA extracted from gastric biopsies

ASP-PCR for 23S rRNA gene was performed with 4 primers described previously by Furuta et al. (2007). The primer sequences were FP-1 (5′-TCGAAGGTTAAGAGGATGCGTCAGTC-3′), FP2143G (5′-CCGCGGCAAGA CAGAGA-3′), RP-1(5′-GACTCCATAAGAGCCAAAGCCCTTAC-3′), and RP2142G(5′-AGTAAAGGTCCACGGGGTATTCC-3′). ASP-PCR was performed with KOD Xtreme™ Hot Start DNA Polymerase (Toyobo, Osaka, Japan) and obtained a band of 320 bp for both wild type (wt) and mutant, 238 bp for A2142G mutation, and 118 bp for A2143G mutation.

The mix for KOD Xtreme™ Hot Start DNA Polymerase was performed using 2.4 μL of distilled water, 10 μL of 2X reaction Xtreme buffer, 4 μL of dNTPs (200 μmol/L each), 0.3 μmol/L each primer, 0.4 μL KOD Xtreme Hot Start DNA polymerase, and 2 μL of DNA template. The amplification was conducted under the following conditions: 1 cycle at 94 °C for 2 min; 40 cycles of 98 °C for 10 s, 65 °C for 30 s, 68 °C for 20 s, with a final extension at 72 °C for 2 min.

2.6. ASP-PCR for to determine gyrA mutation of H. pylori from DNA extracted from gastric biopsies

ASP-PCR for the *gyrA* gene was performed with the primers described previously by Nishizawa et al. (2007) (Table 1). Three new primers were designed to determine mutations at positions A260T, T261C, related with N87I mutation in the GyrA protein. The design of the primers was based on the *gyrA* sequence of *H. pylori* 26695 (Table 1).

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