Detection of Clarithromycin-Resistant *Helicobacter pylori* in Stool Samples

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Received 29 January 2003/Returned for modification 30 March 2003/Accepted 24 May 2003

The recognition of the role of *Helicobacter pylori* in gastric diseases has led to the widespread use of antibiotics in the eradication of this pathogen. The most advocated therapy, triple therapy, often includes clarithromycin. It is well known that clarithromycin resistance is one of the major causes of eradication failure. The development of a rapid noninvasive technique that could easily be performed on fecal samples and that could also provide information about the antibiotic resistance of this microorganism is therefore advisable. Previous findings have demonstrated that clarithromycin resistance is due to a single point mutation in the 23S rRNA. All the mutations described have been associated with specific restriction sites, namely *Bsa*I (A2143G), *Mbo*II (A2142C/G), and *Hha*I (T2717C). On this basis we have developed a new method, a seminested PCR, allowing screening for clarithromycin resistance of *H. pylori* directly on stool samples. This method furnished a 783-bp fragment of the 23S rRNA, which was subsequently digested by *Mbo*II, *Bsa*I, and *Hha*I, in order to identify single point mutations associated with clarithromycin resistance. Of a total of 283 stool samples examined, 125 were *H. pylori* positive and two of them were shown to contain clarithromycin-resistant strains due to the presence of a mutation at position 2717, whereas no PCR products contained mutations at position 2142 or 2143. In order to evaluate the reliability of the new system, we compared the results of restriction analysis of the PCR products with the MICs shown by the *H. pylori* isolates by culturing gastric biopsies from the same patients.

The clinical relevance of *Helicobacter pylori* infection has led to the development of several diagnostic methods, especially noninvasive ones (4, 12, 17, 21, 24). To date most of them are PCR-based methods that are directly performed on gastric biopsy samples (9, 13, 14, 16). The usefulness of these methods remains the rapidity in detection of this bacterium, but neither eliminates the need for gastric endoscopy or gives complete predictive information about antibiotic resistance in *H. pylori* (1, 14, 26). Moreover, recognition of the role of *H. pylori* in gastric diseases has led to the widespread use of antibiotics in the eradication of this pathogen.

The most advocated therapy, triple therapy, often includes clarithromycin (1, 11). However, failure to eradicate *H. pylori* occurs due to resistance to antibiotics, in particular to macrolides, whose prevalence is increasing (1, 2, 10). The mechanisms of clarithromycin resistance have been elucidated and consist of a mutation in the functional domains of the 23S rRNA in *H. pylori*, which is most frequently located in domain V, as well as that in domain VI. In particular, the main 23S rRNA mutations are an adenine-to-guanine transition at positions 2142 and 2143, an adenine-to-cytosine transversion at position 2142, and a thymine-to-cytosine transition at position 2717. All these mutations confer resistance by altering the ribosome target (7, 25, 26). These single point mutations also generate specific restriction sites, namely *Bsa*I, *Mbo*II, and *Hha*I, which can be used for the rapid screening of clarithromycin resistance.

However, at present the only way to assess clarithromycin resistance is either by testing *H. pylori* isolates cultured from gastric biopsies or by PCR methods performed directly on gastric specimens (7, 14, 26). In both cases, this means subjecting patients to invasive procedures such as gastric duodenoscopy. The aim of the present study was the rapid assessment of clarithromycin resistance, in particular the development of a noninvasive technique combining the advantage of use on stool samples (with the excellent performance of the PCR) with rapid screening of clarithromycin resistance of *H. pylori* from infected patients.

**MATERIALS AND METHODS**

**Patients.** A total of 283 patients (253 adult patients with a median age of 45 years [range, 22 to 68 years] and 30 pediatric patients with a median age of 5 years) presenting with upper gastrointestinal symptoms (severe dyspeptic symptoms such as discomfort or pain or both, centered in the upper abdomen) were enrolled in the study. No patients had undergone eradication therapy.

All patients underwent upper gastrointestinal endoscopy in order to confirm, with the traditional culturing method, the results of restriction analysis of the PCR products. In particular, three biopsies for each patient, taken from the antrum, the gastric body, and the duodenal mucosa with a disinfected endoscope, were placed in 0.1 ml of sterile saline solution and microbiologically processed within 2 h. A rapid test for the detection of urease activity was also performed on biopsy samples (8, 16).

**Bacteria and culture conditions and antimicrobial susceptibilities of the isolates.** Biopsy samples were cultured and *H. pylori* isolates were identified as previously described by us (6, 7). All the *H. pylori* isolates obtained by culturing biopsy samples were tested for antimicrobial susceptibility with the agar dilution
methodology approved by National Committee for Clinical Laboratory Standards (19, 21). Isolates were classified as clarithromycin resistant if the MIC exceeded 1 μg/ml (18, 19). In the study, positive controls for the 2142 and 2143 single point mutations (associated with a high level of clarithromycin resistance) were included. For each strain, combined with the seminested PCR, the presence of the mutation was confirmed by sequence analysis.

**Stool sample collection and processing.** Fresh fecal specimens were collected from all patients enrolled in the study. All samples were analyzed within 2 h of collection; otherwise they were held at −20°C until processing.

**DNA purification from stool samples.** Rapid DNA purification from both fresh and frozen fecal samples was performed with the QIAamp DNA stool minikit (Qiagen) according to the manufacturer’s instructions. In order to optimize DNA purification yield, 220 mg of fecal samples was used as starting material. Following lysis, 10 μl of eluate was obtained from each sample. Each eluate was then purified, eliminating any RNA residue, by the addition of 5 μl of RNase A (10 mg/ml) followed by incubation at 60°C for an hour (7). Then 5 μl of eluate was used for the seminested PCR, while the remainder was stored at −20°C.

**H. pylori detection in stool samples: PCR conditions.** *H. pylori* was detected with the seminested PCR amplification method for a portion of the 23S rRNA gene. The reaction was performed directly on eluates obtained from stool sample purification. Two microliters of QIAamp-purified DNA was added to each PCR amplification reaction. The reaction was performed with a couple of outer primers whose nucleotide sequence was derived from a known sequence of the 23S rRNA gene: HP1 forward (5′-CCACACGCGATGGTGTCCAG-3′) (1820 to 1840); GenBank accession number U27270) and Hp2 reverse (5′-TGT-GTA-GCT-ACC-CAG-CGA-TGC-TC-3′) (2811 to 2790; GenBank accession number U27270) in the first reaction.

Amplification reaction mixtures (50 μl) contained 10 mM Tris-Cl (pH 9.0), 50 mM KCl, 1.5 mM MgCl₂, 2.5 U of Taq DNA polymerase (Amersham Pharmacia, Biotec, Piscataway, N.J.), 200 μM deoxynucleoside triphosphate mix, 12.5 pmol of each primer, and 5 μl of eluate from stool sample. Amplification was carried out in a DNA Thermal Cycler 9700 (Perkin-Elmer Corporation, Norwalk, Conn.). Thirty cycles, each consisting of 45 s at 95°C, 45 s at 65°C, and 45 s at 72°C, were performed after 2 min of denaturing at 95°C. Cycles were followed by a final elongation at 72°C for 4 min. The PCR product resulted in 993 bp. Then 2 μl of the PCR product obtained from the first reaction was added to the mixture of the second PCR employing a second set of primers (one of which represents an inner sequence of the first PCR product): HP4 forward (2028 to 2048; 5′-GTGCGGTAAAATTACGACCTG-3′) and HP2 reverse. The second PCR, utilizing both the same reaction mixture and the same PCR conditions as described above, furnished a 783-bp PCR product.

A 10-μl portion of the PCR product was then analyzed by electrophoresis in a 2.0% agarose gel in Tris-acetate-EDTA (TAE) buffer and stained with ethidium bromide. The PCR products were examined in parallel with molecular size markers, the Gene Ruler 100-bp DNA ladder size markers (MBI Fermentas, Foster City, Calif.). DNA sequences were determined with an ABI Prism 3100 (Applied Biosystems, Foster City, Calif.).

**Sensitivity and specificity of seminested PCR.** The PCR method yielded the expected amplicons of 783 bp for 125 fecal specimens (of a total of 283 stool samples from different patients examined). These results were confirmed by the culturing method: 125 cultures positive for *H. pylori* were obtained from the same patients whose fecal samples had furnished an amplified product. In contrast, no fragment was obtained from fecal samples from patients who were *H. pylori* negative with the culture method. The results of the amplification assay, performed on all negative samples, excluded the possibility of false-negative stool samples. All negative samples, analyzed by a β-actin PCR, were shown to be free of any inhibitors, furnishing good PCR products (Fig. 1).

**RESULTS**

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The sensitivity of the PCR method was evaluated by testing a fecal sample (previously demonstrated to be *H. pylori* free) artificially inoculated with a suspension of cultured *H. pylori* containing 15,000 CFU, 1,500 CFU, and 150 CFU. The detection limit of the PCR method was 1,500 CFU; no PCR product was obtained from fecal samples inoculated with 150 CFU of *H. pylori*. Moreover, storage at −20°C and subsequent thawing did not affect the ability of the PCR method to provide the amplicon.

The specificity of our method has been proved by performing the specificity assay, by which we have demonstrated that the PCR did not produce amplicons for any of the control strains, some of which can be present in the gut (Fig. 2). This
Restriction analysis of 125 PCR products showed that only two samples contained clarithromycin-resistant *H. pylori*, their amplified products being susceptible to *Hha*I digestion. A positive control for the single point mutation at either position 2142 or 2143 was added to the study, but none of the 125 amplicons contained these mutations. Figure 3 is a comparison of restriction analysis (with *Bsa*I, *Mbo*II, and *Hha*I) of some amplicons corresponding to the susceptible phenotype as well as the restriction pattern obtained from clarithromycin-resistant phenotypes defined by our method or introduced as positive controls. The study of antimicrobial susceptibilities performed on *H. pylori* isolates obtained by culturing biopsy samples from the same patients whose stool specimens had previously been analyzed by PCR confirmed the results of the restriction analysis. None of the 125 isolates expressed a high level of clarithromycin resistance, while only two strains were clarithromycin resistant, expressing a MIC of 1 μg/ml (18, 19, 21).

**DISCUSSION**

Most of the guidelines published on the management of *H. pylori* infection do not recommend performing susceptibility testing before treating patients for whom *H. pylori* infection has been diagnosed for the first time (5, 23). The main reason is probably that *H. pylori* culture as well as antimicrobial susceptibility studies are difficult to perform as well as labor intensive. Moreover, although the culture method allows antimicrobial susceptibility testing for several antibiotics, only the susceptibilities of macrolides and, in particular, of clarithromycin are really useful since the last is a major predictor of treatment failure (17). Therefore, detection of clarithromycin-resistant *H. pylori* will facilitate the choice of an appropriate eradication regimen (1).

In the past, Versalovic et al. developed a method (utilizing rapid restriction analysis of the amplicon obtained from *H. pylori*) which avoided the antimicrobial susceptibility testing of the *H. pylori* isolates, and subsequently Marais et al. and Oleastro et al. developed systems which could detect either *H. pylori* or clarithromycin resistance of the microorganism directly on gastric biopsy by a PCR method (14, 20, 26). Moreover, Monteiro et al. developed a system to detect *H. pylori* directly on stool samples by PCR (16, 17). These represent a great effort to change the *H. pylori* diagnostic algorithm, particularly because the authors propose a strategy to remove the inhibitors present in fecal material that represent the main problem in performing the amplification procedure in stool specimens (4, 17). However, to date none of the methods described have combined the benefits of avoiding an invasive procedure in the assessment of the *H. pylori* status of the patient with the usefulness of rapidly obtaining complete information concerning the antimicrobial susceptibilities of the microorganism, particularly those regarding clarithromycin resistance. This was the goal of the present work.

We have in fact developed a simple, rapid, and cost-effective procedure which can detect *H. pylori* in patient stool specimens with good sensitivity and evaluate the clarithromycin resistance of the microorganism. Also in our case, removal of inhibitors has been our main objective and was achieved with a commercial system of purification, which is simple to use, extremely...
patterns of two samples containing Helicobacter pylori. In the part of the figure illustrating HhaI digestion, lanes c and g correspond to the restriction patterns of two samples containing H. pylori with the T2142C transition in the 23S RNA gene (low-level Clr phenotype). In the part of the figure illustrating BsaI digestion, lane h corresponds to the restriction pattern of a positive control for H. pylori with a mutation at position 2143. In the part of the figure showing MboII digestion, lane h correspond to the restriction pattern of a positive control for H. pylori with a mutation at position 2142.

In conclusion, our PCR detection of H. pylori in stool specimens is reliable and easy to perform and can provide additional information specifically related to the macrolide susceptibility of the microorganism. Therefore, the extensive use of this method not only reduces the use of invasive procedures in order to isolate H. pylori infecting the patient, but also allows direction of the first eradication therapy or evaluation of the outcome of previous eradication regimens (with a consequent reduction in cost). Finally, the advisable widespread use of this procedure will allow a reduction in the use of gastro-duodenal endoscopy, which is expensive and not always advisable in some patients, such as the pediatric population.

ACKNOWLEDGMENTS

We gratefully acknowledge the excellent technical collaboration of Francesca Capalbo, Oriana Cicchetti, Alessandro Mauti, and Marco Pelliccioni. We thank Alison Inglis for helpful linguistic revision of the manuscript.

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