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

Carla Fontana,1,2* Marco Favaro,3 Antonio Pietroiusti,4 Enrico Salvatore Pistoia,1 Alberto Galante,4,5 and Cartesio Favalli1,2

Department of Experimental Medicine and Biochemical Sciences,1 Department of Biology,3 and Department of Internal Medicine,4 “Tor Vergata” University of Rome, and Clinical Microbiology Laboratories, Policlinic of Tor Vergata,5 00133 Rome, and Clinica San Raffaele, Velletri,2 Italy

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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 (A2142G), 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*II, 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 used for detection of the A2142C/G mutation, and digestion was performed at 30°C for 1 h according to the manufacturer’s instructions. The digestion patterns of the PCR products were analyzed by electrophoresis in 2.0% agarose gel, stained with ethidium bromide, in TAE buffer, and compared with the reference control (H. pylori ATCC 43504) as well as with the Gene Ruler 100-bp DNA ladder size markers (MBI Fermentas).

Sensitivity of the new method. In order to evaluate the sensitivity of the new method for the rapid detection of clarithromycin resistance in fecal samples, a stool sample previously demonstrated to be H. pylori free was artificially inoculated with H. pylori obtained from a 3-day-old culture on nonselective medium. In particular, a suspension of cultured H. pylori, equivalent to 0.5 opacity on the MacFarland scale (corresponding to 1.5 × 10^6 CFU/ml) was prepared in sterile H2O, and 10 μl of this suspension, 10 μl of a 10-fold dilution (15,000 CFU) of this suspension (1,500 CFU), and finally 10 μl of a 100-fold dilution of the suspension (150 CFU) were added to fecal samples (each of them weighing 220 mg). Following the additions, the samples were better homogenized and analyzed following the PCR protocol described above. In order to investigate the stability of H. pylori DNA during storage, samples which were optimal for the amplification method were also frozen for a day and then thawed and subsequently analyzed by PCR.

Specificity assay. To avoid any possible false-positive results caused by our technique, we performed an experiment to control for specificity. The assay utilizes crude lysates from strains which can colonize the gastric mucosa, such as Helicobacter heilmannii ATCC 49286, Campylobacter jejuni subsp. jejuni ATCC 33291, and other strains which can be present in fecal samples, such as Escherichia coli ATCC 25922 and Staphylococcus aureus ATCC 25923. Crude saline lysates were prepared as reported above, and also in this case 10 μl of supernatant of this crude lysate was added to each PCR amplification reaction. The PCR protocol was that described previously.

Amplification assay. To exclude the possibility that some stool specimens could be negative due to the presence of inhibitors, all negative samples were subjected to a second PCR assay with the purpose of amplifying the conserved gene for human β-actin (22). The PCR was performed according to Ponte et al. and performed directly on 2 μl of eluate obtained from stool sample purification of QIAamp-purified DNA.

RESULTS

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).

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, or 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
result allowed us to exclude the possibility that a fecal specimen could be a false-positive sample.

Reliability in the detection of clarithromycin-resistant \textit{H. pylori}. Of a total of 283 fecal samples examined, 125 furnished amplified products of 783 bp. In order to highlight mutations at positions 2143, 2142, and 2717 in the 23S rRNA, the PCR products underwent endonuclease restriction analysis with \textit{BsaI}, \textit{MboII}, and \textit{HhaI}. Digestion by \textit{HhaI} furnished three fragments of 515, 168, and 100 bp in the presence of the T2717C transition (associated with a phenotype of low-level resistance to clarithromycin), and only two fragments of 615 and 168 bp in the absence of this mutation. \textit{MboII} digestion furnished two fragments of 670 and 112 bp in the presence of a mutation at position 2142 and a single fragment corresponding to the size of the PCR product (783 bp) in the case of the wild-type phenotype. Finally, the mutation at position 2143 highlighted by \textit{BsaI} digestion produced two fragments of 671 and 113 bp. Therefore, a single fragment of 783 bp, after \textit{BsaI} digestion, demonstrates the absence of the mutation at 2143 in the 23S rRNA (7, 26).

Restriction analysis of 125 PCR products showed that only two samples contained clarithromycin-resistant \textit{H. pylori}, their amplified products being susceptible to \textit{HhaI} 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 \textit{BsaI}, \textit{MboII}, and \textit{HhaI}) 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 \textit{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 \(\mu\)g/ml (18, 19, 21).

**DISCUSSION**

Most of the guidelines published on the management of \textit{H. pylori} infection do not recommend performing susceptibility testing before treating patients for whom \textit{H. pylori} infection has been diagnosed for the first time (5, 23). The main reason is probably that \textit{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 \textit{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 \textit{H. pylori}) which avoided the antimicrobial susceptibility testing of the \textit{H. pylori} isolates, and subsequently Marais et al. and Oleastro et al. developed systems which could detect either \textit{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 \textit{H. pylori} directly on stool samples by PCR (16, 17). These represent a great effort to change the \textit{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 \textit{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 \textit{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
rapid, and requires only a few steps, reducing the risk of contamination by foreign DNA (4). The second aspect in the development of this procedure has been the combination of knowledge concerning all restriction sites associated with 23S rRNA mutations (those associated with high and low resistance phenotypes) and therefore clarithromycin resistance (1, 22, 26). The result has been rapid endonuclease restriction analysis of the amplicons which is easy to perform and does not present any difficulty in interpretation.

In conclusion, our PCR detection of \textit{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 \textit{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 gastroduodenal endoscopy, which is expensive and not always advisable in some patients, such as the pediatric population.

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REFERENCES


