Detection of *Helicobacter pylori* in Stool Specimens by PCR and Antigen Enzyme Immunoassay

ATHANASIOS MAKRISTATHIS,1,1 EVA PASCHING,1 KURT SCHÜTZE,2 MARGIT WIMMER,1 MANFRED L. ROTTER,1 AND ALEXANDER M. HIRSCHL1

Department of Clinical Microbiology, Hygiene Institute of the University of Vienna,1 and Medical Department I, Hanusch Hospital,2 Vienna, Austria

Received 21 January 1998/Returned for modification 16 March 1998/Accepted 11 June 1998

A highly sensitive seminested PCR assay to detect *Helicobacter pylori* DNA in feces was developed. PCR with stool specimens and a novel antigen enzyme immunoassay (EIA) for *H. pylori* detection in feces were evaluated as diagnostic tools and in follow-up with samples from 63 infected and 37 noninfected persons. Infected individuals received eradication therapy followed by endoscopic follow-up 35 days after the start of treatment. At that time, a second stool specimen was obtained from 55 of these patients. Before eradication, the sensitivity of PCR was 93.7% and that of EIA 88.9%. Specificities were 100 and 94.6%, respectively. Of the 55 follow-up specimens, 41 originated from patients from whom *H. pylori* had been eradicated. Of these, 21 were still positive by PCR and 13 were positive by EIA, indicating that 1 month may be too short a period for follow-up evaluation of stool specimens by these tests.

*Helicobacter pylori* has been associated with gastritis, peptic ulcers, and gastric carcinoma (2, 5). Infection occurs mainly in childhood and shows lifelong persistence in the gastric mucosa (1, 7). There is evidence that infected individuals excrete *H. pylori* in feces, since the pathogen could be detected in stool specimens by PCR or even culture (8, 10, 13). PCR is a powerful technique for the detection of target DNA in various clinical specimens, but its application to fecal specimens has been limited due to the presence of substances inhibiting the reaction. The aim of the present study was to develop a sensitive PCR protocol to detect *H. pylori* DNA in feces. Since it is noninvasive, this method for diagnosing infection would be of particular importance for very young pediatric patients. Furthermore, within the framework of a clinical trial, the purpose of this study was to determine the value of PCR of stool samples as both a diagnostic tool and a follow-up method after eradication therapy. In addition, a new antigen enzyme immunoassay (EIA) for detection of *H. pylori* in feces was evaluated with respect to sensitivity, specificity, and follow-up.

Initially, stool specimens of 10 noninfected individuals—as proven by the [13C]urea breath test and serology—were used to develop an extraction and purification protocol, yielding DNA virtually free of PCR inhibitors. To evaluate the sensitivity of the method, each specimen was spiked with *H. pylori* (CCUG 38770) in a range of between 100 and 100,000 bacteria/g of stool. For this purpose, *H. pylori* was grown under microaerobic conditions at 37°C in *Brucella* broth (Difco Laboratories, Detroit, Mich.) containing 7% fetal calf serum. Then, serial dilutions were produced with lysis buffer (50 mM Tris-HCl, 0.5% Tween 20, 0.5% Triton X-100 [pH 8.0]; Qiagen, Hilden, Germany), and stool was added to each dilution. Furthermore, sensitivity was evaluated by adding dilutions of purified *H. pylori* DNA to the DNA extract of each specimen.

For extraction of DNA from stool specimens, the following protocol was used. A 100-mg amount of feces was thoroughly suspended in 1 ml of lysis buffer (see above), and the suspension was centrifuged (60 × g, 2 min). Debris was discarded. The supernatant was boiled (10 min) to ensure complete lysis and was centrifuged (10,000 × g, 10 min) to remove particles. The DNA solution was incubated (30 min, 37°C) in the presence of RNase A (0.2 mg/ml; Qiagen) and proteinase K (0.5 mg/ml; Qiagen). Then, a one-third volume of guanidine-HCl buffer (3 M guanidine-HCl, 20% Tween 20 [pH 5.5]; Qiagen) was added, and the specimen was incubated for 30 min at 50°C. DNA was purified by column chromatography (Genomic Tip 20/G; Qiagen) according to the manufacturer’s protocol, followed by chloroform, phenol-chloroform-isoamylalcohol, and chloroform-isoamylalcohol extractions of the eluate. In a further purification step, DNA was then concentrated with a Microcon 100 filter (Amicon, Inc., Beverly, Mass.).

PCR was based on the DNA sequence of a species-specific protein antigen with an M, of 27,000. Primary amplification reaction was performed by a protocol described elsewhere (4). The reaction was followed by a seminested PCR developed in our laboratory with the upstream primer 5‘-TGGGCGTGTCTATTGACAGCGAGC-3’ of the primary PCR and the nested primer 5‘-TGATCATCAGCATGTACTTCCATGTTTTT-3’. These oligonucleotides are identical to residues 474 to 496 and 682 to 652, respectively, of the published sequence (11).

Reactions were performed with a volume of 50 μl with TRIÖ-Thermoblock (Biometra, Göttingen, Germany) for thermal cycling. Primary PCR product (0.5 μl) was added to a reaction mixture consisting of the four deoxynucleotides (Boehringer, Mannheim, Germany) at 100 μM each, 0.1 μM each primer (Codon Genetic Systems, Vienna, Austria), and 0.5 U of the Taq DNA polymerase (Boehringer) in a standard PCR incubation buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 2.5 mM MgCl₂, 0.001% gelatin). Amplification in the seminested PCR consisted of an initial denaturation step at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 68°C for 1 min, and extension at 72°C for 45 s. After the last cycle, the mixture was incubated at 72°C for 5 min. The amplification product was analyzed by electrophoresis on a 1.5% agarose gel by a standard protocol (12), and a band at 209 bp was considered a positive PCR result. Confirmation of the PCR result was performed by dot blot hybridization.
Briefly, 1 μl of the postamplification reaction mixture was transferred on a nylon membrane (Hybond-N+; Amersham International plc, Buckinghamshire, United Kingdom) and hybridized according to the manufacturer’s protocol. The purified product of the primary PCR (with \textit{H. pylori} DNA extract as the template) was biotinylated (Biotin-High Prime; Boehringer) and used as a DNA probe. Detection was performed by chemiluminescence (SuperSignal Nucleic Acid kit; Pierce, Rockford, III.).

According to the extraction protocol described above, boiling of samples and discarding of insoluble material as well as additional DNA purification with organic solvents and a Microcon filter resulted in a sensitivity of the assay that was approximately fivefold higher. Furthermore, seminested PCR was approximately 10 times more sensitive than primary PCR. By this protocol, all spiked stool specimens were positive when they contained 1,000 bacteria/g (equivalent to DNA of 6 bacteria in the primary PCR), which almost corresponds to the sensitivity of the assay when bacterial suspensions without stool were used.

Recently, a novel enzyme immunoassay (EIA; Premier Platinum HpSA; Meridian Diagnostics Inc., Cincinnati, Ohio) utilizing immunoaffinity-purified polyclonal anti-\textit{H. pylori} rabbit antibody adsorbed to microwells for detection of \textit{H. pylori} antigens in feces has been developed. To evaluate antigen EIA (performed as indicated by the manufacturer) as well as stool PCR in a clinical study, fecal specimens of 63 \textit{H. pylori}-infected adults, all of whom suffered from duodenal ulcers, were examined by both tests. Following endoscopic diagnosis of duodenal ulcers and confirmation of \textit{H. pylori} infection by histology and culture, the patients received a 7-day treatment with 750 mg of amoxicillin three times a day (t.i.d.) combined with 500 mg of metronidazole t.i.d. In addition, the patients were given 20 mg of omeprazole every morning over the same period. A clinical follow-up evaluation was done 7 days after the start of treatment. An endoscopic follow-up for eradication control was performed 35 days after the start of treatment, and gastric biopsy specimens were examined for the presence of \textit{H. pylori} by histology and culture. At that time, a second stool specimen was obtained from 55 of the 63 patients.

To evaluate specificity as well as positive and negative predictive values (PPVs and NPVs, respectively) of both tests, feces from 37 noninfected individuals—as proven by the \textsuperscript{[13C]}urea breath test and serology—were also tested. This number was chosen to give a positivity of 63% in the study population (63 infected and 37 noninfected persons), which corresponds to the prevalence of \textit{H. pylori} infection in our routine \textit{H. pylori} laboratory diagnostic performed by culture, serology, and the \textsuperscript{[13C]}urea breath test.

All stool specimens were stored at −70°C until both tests were performed. No sequences other than the expected product were amplified in the seminested PCR (Fig. 1). Fifty-nine (93.7%) of the 63 fecal specimens obtained from the patients before treatment were positive by \textit{H. pylori} stool PCR, and 56 (88.9%) were positive by EIA (Table 1). Only 53 of the 63 specimens (84.1%) were positive by both tests. However, for samples considered positive by either PCR or EIA, 62 of the 63 fecal specimens from \textit{H. pylori}-infected adults were positive (98.4%). Of the 37 fecal specimens of noninfected individuals, none were positive by PCR and only 2 were positive by EIA, suggesting specificities of 100 and 94.6%, respectively. The PPVs were 100% by PCR and 96.6% by EIA. The NPVs were 90.2% by PCR and 83.3% by EIA.

With respect to the 55 patients delivering stool specimens at the date of the follow-up endoscopy, \textit{H. pylori} was eradicated in 41. Fourteen patients still had positive \textit{H. pylori} results by histology and culture. Stool PCR gave positive results for 13 (92.9%) and antigen was detected in feces by EIA for 12 (85.7%) of these 14 patients (Table 1). Only 11 (78.6%) fecal specimens were positive by both tests; however, all (100%) of the 14 specimens were considered positive when the results of the two tests were taken together. Surprisingly, PCR and EIA still gave positive results for 21 and 13, respectively, of the 41 patients from whom \textit{H. pylori} had been successfully eradicated (Table 1), indicating that 1 month may be too short a period for follow-up evaluation of stool specimens by EIA (specificity, 68.3%; PPV, 48%; and NPV, 93.3%) and especially by PCR (specificity, 48.8%; PPV, 38.2%; and NPV, 95.2%).

Accurate detection of \textit{H. pylori} in the feces of infected patients is of considerable importance, in particular for very young pediatric patients, who should preferentially be investigated by noninvasive methods. The \textsuperscript{[13C]}urea breath test is a well-established, highly sensitive, and specific noninvasive method (3). However, expensive specialized instrumentation is required, and performance of the test has been associated with several disadvantages, especially in infants.

The stool PCR method described in this paper appears to be highly sensitive and specific (before eradication). This could be achieved by the seminested PCR and also by the removal of potential inhibitory substances present in feces. Elimination of these substances was done by centrifugation steps, column chromatography, phenol-chloroform extraction, and a concen-

![Table 1](https://example.com/Table1.png)

**Table 1. Results of PCR and EIA compared to \textit{H. pylori} status before and after eradication therapy.**

<table>
<thead>
<tr>
<th>Method and result</th>
<th>No. of individuals with indicated \textit{H. pylori} status</th>
<th>Before therapy (n = 100)</th>
<th>After therapy (n = 55)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive(^a)</td>
<td>Negative(^a)</td>
<td>Positive(^b)</td>
</tr>
<tr>
<td>PCR</td>
<td>Positive</td>
<td>59</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>4</td>
<td>37</td>
</tr>
<tr>
<td>EIA</td>
<td>Positive</td>
<td>56</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>7</td>
<td>35</td>
</tr>
</tbody>
</table>

\(^a\) \textit{H. pylori} status was proven by histology and culture.

\(^b\) \textit{H. pylori} status was proven by the \textsuperscript{[13C]}urea breath test and serology.
tration step with a Microcon 100 filter. This final step is to remove remaining nonbiological inhibitory substances as well as complex polysaccharides (MW, as much as 80,000), which have been found to be potent PCR inhibitors (9), and allows elimination of time-consuming DNA precipitation.

Although the sensitivity and specificity values of antigen EIA for preeradication specimens are somewhat lower than those for PCR and the high PPV (96.6%) would decrease with lower prevalence of *H. pylori* infection, this novel EIA was shown to be a useful noninvasive diagnostic test which is easy to perform, with only approximately 90 min required for a large number of samples.

Thus, the results of this study suggest that both PCR and antigen EIA are suitable tools for detection of *H. pylori* infection. Moreover, the two tests together are almost as sensitive as histology and culture of biopsy specimens. Persistence of *H. pylori* DNA or antigen in many of the patients evaluated was an unexpected finding, since it is well known that gastric mucosal replacement takes less than 1 week (6). However, it may be thinkable that under certain circumstances (presence of the appendix or diverticulosis), passage of the gastric epithelial cells could be delayed. Nevertheless, this phenomenon still requires clarification. Currently, fecal specimens of successfully treated individuals (children and adults) are being evaluated by both tests for prolonged time periods to determine whether PCR and EIA are appropriate for follow-up investigations and, if so, to establish the optimal time point for their application.

REFERENCES


