# Diagnostic Methods for Detecting Forms and Strains *of Helicobacter Pylori*and Evaluation of Its Eradication

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Diagnostic methods for detecting forms and strains of *Helicobacter pylori* isolated from biopsy specimens of gastric mucosa in 28 patients with duodenal ulcers and evaluation of its eradication were compared. Biopsy specimens from all patients were tested for the presence of *H. pylori* by the urease test, histological method, and PCR with species-specific primers before and after treatment. *H. pylori* infection was detected in all patients before treatment, the mean titer of serum IgG being 36.7±16.6 U/ml. Biopsy specimens positive for *H. pylori* in PCR were subjected to restriction analysis of specific PCR-amplified genes or their fragments. The fingerprint analysis gave electrophoregrams of restriction products amplified fragment of *flaA* gene of *H. pylori* in 7 patients. Differences in restriction maps indicate the presence of 5 *H. pylori* strains in the studied samples.

**Key Words:** *Helicobacter pylori; duodenal ulcer; fingerprint analysis*

*Helicobacter pylori* is one of the most important etiological factors of gastroduodenal ulcers. The *Helicobacter* genus is heterogeneous and includes 19 species, 8 of which persist in the gastric mucosa and 11 in the small intestinal mucosa. In humans only seven *Helicobacter* species occur: *H. pylori, H. heilmannii, H. felis* in the gastric mucosa and *H. cinaedi, H. fennelliae, H. canis, and H. pullorum* in the small intestinal mucosa. Each of these species, including *H. pylori,* includes several strains [4]. All of them produce urease. Many tests for detecting *H. pylori* are based on high urease activity of these bacteria.

Determinants responsible for *H. pylori* virulence are represented by gene *vacA* producing vacuolar cytotoxin, cytotoxin-associated gene *cagA*, flagellar genes *flaA, flaB*, genes encoding urease (*ureA, B, C, D, E, F, G, H, I*), and gene *picB* potentially capable of inducing the production of interleukin-8 by gastric epithelial cells [7]. *H. pylori* strains from patients with gastroduodenal ulcers attract special attention; they express specific proteins: vacuolar toxin (86 kDa) and highly antigenic CagA protein (120-128 kDa) encoded by gene *cagA.*

*H. pylori* strains differ by drug resistance, adhesive specificity, cytotoxin and CagA protein production, and nucleotide sequence of genes (or their fragments) encoding certain *H. pylori* proteins. The most polymorphic are genes *flaA* and *ureA, B, C.* Genetic typing of *H. pylori* strains became possible with the development of molecular diagnosis. A highly sensitive and specific fingerprint analysis is now used for this purpose. Several modifications of this method are known: analysis of amplified DNA by arbitrary primers (RAPD), restriction fragment length polymorphism analysis with specific DNA probes (RFLP), restriction analysis of genomic DNA (genomic REA), and restriction analysis of specific PCR-amplified genes or their fragments (PCR-RFLP) [3]. However all these modifications are based on the use of intact DNA isolated from bacterial culture [5]. Unlike RAPD, RFLP, and genomic REA, the PCR-RFLP method is based on restriction analysis of relatively small PCR pro-

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ducts. This provides a theoretical basis for DNA analysis of *H. pylori* isolated directly from biopsy specimens without preliminary culturing of bacteria [6]. Solution of this problem would make it possible to isolate and differentiate *H. pylori* immediately from biopsy specimens, detect several *H. pylori* strains in a biopsy specimen from one patient, evaluate the resistance of *H. pylori* strains to anti-Helicobacter therapy, detect silent forms and *H. pylori* reinfection.

However, we found no experimental validation for such potentialities in available publications.

The aim of the present study was to evaluate diagnostic potentialities of various methods for detection of differentiated forms and strains of *H. pylori* and to compare the efficiency of the most prevalent protocols of anti-Helicobacter therapy by these methods.

## MATERIALS AND METHODS

Twenty-eight patients with gastroduodenal ulcers (GDU) were examined: 5 women aged 33-57 years (mean age 43.8±10.5 years) and 23 men aged 18-70 years (43.6± 15.7 years). In 14 patients GDU often (more than twice a year) relapsed. Esophagogastroduodenoscopy with spot biopsy from the antral portion of the stomach was carried out with a Pentax FG-29P device and biopsy specimens were stored at -20°C. Preliminary selection of *H. pylori*-positive GDU patients was carried out using quantitative spectrophotometry of urease activity [1,2]. The patients were divided into 2 groups treated by different protocols. Group 1 ($n$=11) received omeprazole (Losec) in a daily dose of 40 mg for 1 week and 20 mg/day for the next 3 weeks and antibiotic therapy (10 days) with clarithromycin (clacide) (250 mg 3 times a day) and metronidazole (0.5 g twice a day). Group 2 ($n$=17) received pyloride (400 mg twice a day for 4 weeks) and antibiotics like in group 1.

Before therapy all patients were tested for *H. pylori* by the urease test, histological method, and PCR [1,2]. Biopsy specimens of all *H. pylori*-positive patients (according to PCR, called below PCR-positive) were then tested by PCR-RFLP [3].

Isolation of DNA from biopsy specimens and PCR were carried out using species-specific primers for *H. pylori* with Lagis kits according to the manufacturer's instructions.

For amplification of *flaA* gene fragment, DNA was isolated from biopsy specimens using InstaGene Matrix (Bio-Rad) carrier. PCR was carried out as follows: 2 min at 94°C, 40 30-sec cycles at 94°C, 1 min at 60°C, 2 min at 72°C, and the final step 5 min at 72°C.

The primers were 5"ATGGCTTTTCAGGTCAA TAC-3' and 5"GCTTAAGATATTTTGTTGAACG-3'. Restriction was carried out with *HhaI* restrictase (Promega) in accordance with the manufacturer protocol in the working concentration of 5 U/20 μl sample.

Twenty-six patients were examined repeatedly (esophagogastroduodenoscopy, urease test for *H. pylori*, histological analysis, and PCR) 1 month and 24 patients 6 months after the end of therapy.

In addition to detection of *H. pylori* in the gastric mucosa, serological tests for antibodies (IgG) to *H. pylori* were carried out in both groups before and 1 and 6 months after therapy. Antibodies to *H. pylori* in the serum were assayed by enzyme immunoassay with MEDAC Diagnostika kits in accordance with the manufacturer's protocol. Antibody titer above 20 U/ml was considered as the diagnostic.

## RESULTS

Specific antibodies are produced in response to *H. pylori* infection. They appear in the blood in the diagnostic titer as early as during weeks 2-3 postinfection and can circulate in the blood long after eradication of *H. pylori.*

*H. pylori* and serum IgG were present before therapy in all patients (Fig. 1), which indicates active immune response to *H. pylori* antigens. Intensive anti-Helicobacter therapy led to complete eradication of *H. pylori* in the majority ($n$=21) of patients (according to PCR), but 5 patients remained *H. pylori*-positive. We therefore evaluated antibody titers in PCR-positive and PCR-negative patients separately.

A trend to a decrease in the mean antibody titer was observed in both groups 1 month after therapy, but the diagnostic titer of IgG persisted. Antibodies in diagnostic titers were detected in PCR-positive patients for 6 months after therapy presumably due to the

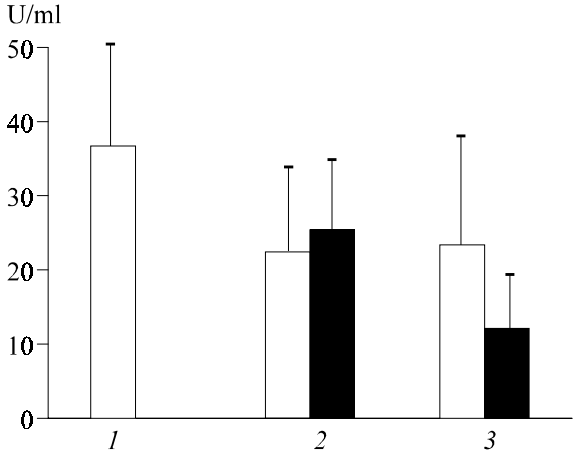


**Fig. 1.** Serum IgG to *Helicobacter pylori* in *H. pylori*-positive (light bars) and negative (dark bars) patients according to PCR results. *1*) before therapy; *2*) 1 month after therapy; *3*) 6 months after therapy. A trend to decrease of anti-Helicobacter IgG in PCR-negative patients: $R^2$=0.997.

**TABLE 1.** Detection of *H. pylori* (Number of Patients) in Gastric Mucosa Biopsy Specimens by Urease Activity (Numerator) and PCR (Denominator)

| Therapy | Before therapy | After therapy | Follow-up results, months | |
|---|---|---|---|---|
| | | | 1 | 6 |
| Omeprazole (*n*=11) | 11/11 | 1/1 | 1/2 (11) | 1/7 (9) |
| Pyloride (*n*=17) | 17/17 | 1/3 | 1/3 (15) | 1/8 (15) |

**Note.** Number of patients examined repeatedly is given in parentheses.

presence of specific *H. pylori* antigens in the silent (dormant) state. By contrast, in PCR-negative patients the level of antibodies to *H. pylori* decreased still more in comparison with the diagnostic titer (Fig. 1). There was a clear-cut linear relationship between the decrease of antibody level and period elapsed after anti-Helicobacter therapy.

The serological method detects specific antibodies to *H. pylori* indicating contact and infection. The diagnostic titer of antibodies can persist for a long time (about 3 months) even after effective eradication treatment.

In group 1 esophagogastroduodenoscopy detected duodenal ulcers in 10 of 11 patients. One patient was included into the group without endoscopically detected ulcer because of frequent relapses of GDU and high degree of *H. pylori* propagation. After therapy the ulcers healed in all 10 patients. According to the urease test, histological method, and PCR, *H. pylori* were completely eradicated. In the Helicobacter-positive patient without ulcer *H. pylori* were not eliminated despite antibiotic therapy. Moreover, very high urease activity persisted in the gastric mucosa, suggesting the resistance of this *H. pylori* strain to clarithromycin and metronidazole.

In group 2 endoscopic examination showed duodenal ulcers in all 17 patients. After therapy ulcers healed in all patients. According to PCR, urease test, and histological analysis of biopsy specimens, *H. pylori* were eliminated in 14 of 17 patients. No eradication was attained in 3 patients. High urease activity and positive PCR signal were observed in one female patient, suggesting resistance of *H. pylori* strain to anti-Helicobacter therapy. Two patients remained PCR-positive, but no urease activity of *H. pylori* was detected in biopsy specimens from these patients, which can indicate that the microorganism transformed into dormant (inactive) forms.

Repeated examination of 11 patients in group 1 one month after therapy showed clinical endoscopic remission of GDU in all of them. High urease activity and PCR signal persisted in gastric mucosa biopsy specimens from the patient with resistant *H. pylori* strain (Table 1). Despite the absence of ulcer, one more PCR-positive patient was detected in this group, probably due to reinfection.

Of 15 patients of group 2 repeatedly examined after 1 month, duodenal ulcer did not heal in only one patient with resistant *H. pylori* strain which was detected by the urease test, histological method, and PCR. In two other PCR-positive patients *H. pylori* was still detected only by PCR 1 month after therapy (Table 1).

After 6 months, 9 patients in group 1 and 15 in group 2 were examined. Esophagogastroduodenoscopy showed no duodenal ulcers. The patient not responding to anti-Helicobacter therapy (group 1) did not come for check-up. In 1 patient *H. pylori* (active form) was detected by the urease test and PCR. The number of PCR-positive patients without urease activity increased; a similar trend was observed after 6 months in group 2 (Table 1).

Hence, urease test does not allow reliable evaluation of the efficiency of eradication therapy, because in some patients *H. pylori* was not completely eliminated after anti-Helicobacter therapy but was transformed into dormant forms. In such cases *H. pylori* can be more often detected by PCR, which is more sensitive and specific than other diagnostic methods. Therefore urease test for detection of *H. pylori* is efficient only before anti-Helicobacter therapy.

The number of PCR-positive patients increased 1 month after therapy and still more after 6 months. Reinfection or activation and multiplication of silent *H. pylori* are possible. We can answer this question only after identification of microorganism strains.

Electrophoregrams of restriction products of *H. pylori* gene *flaA* amplified fragment were obtained for 7 of 28 patients; 5 *H. pylori* strains were detected. Small amount of DNA samples isolated from biopsy specimens can be due to destruction of *H. pylori* DNA during storage (freezing and defrosting) and/or effects of endogenous nucleases. The loss of capacity to amplification of DNA isolated from biopsy specimens

stored for a long time and used repeatedly confirms this hypothesis.

Preliminary data indicate that the PCR-RFLP fingerprinting identifies in some cases *H. pylori* strains directly in the gastric mucosa biopsy specimens. We believe that further studies aimed at optimization of the conditions of the method will essentially improve its efficiency for typing of *H. pylori* in gastric mucosa biopsy specimens. The use of PCR-RFLP will help to disclose the relationship between failure of anti-Helicobacter therapy and presence of resistant or dormant *H. pylori* and individually predict the outcome of therapy. In addition, this method can help to detect the cause of repeated detection of *H. pylori*: infection with a new strain or activation of the agent from the dormant form.

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