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# Diagnosis of *Helicobacter pylori* Infection by PCR: Comparison with Other Invasive Techniques and Detection of *cagA* Gene in Gastric Biopsy Specimens

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**A PCR assay for the detection of *Helicobacter pylori* in gastric biopsy specimens with specific primers for *ureC* gene amplification (herein referred to as *ureC* PCR) was compared with other routine invasive methods (culture, the rapid-urease test, and Giemsa staining of histological sections) with samples from a group of 104 consecutive dyspeptic patients. Bacteria were found in 40 (38.5%), 38 (36.5%), 36 (34.6%), and 35 (33.7%) of the patients by *ureC* PCR, culture, the rapid-urease test, and Giemsa stain, respectively. Sixty-three patients had negative cultures, negative histological examinations, and negative rapid-urease test results, and 61 of these patients were also negative by *ureC* PCR. *ureC* PCR detected *H. pylori* in two culture-negative patients. In parallel, a PCR-based assay to detect the *H. pylori* cytotoxin-associated antigen (*cagA*) gene, a putative virulence gene, was also developed. To assess the likelihood of detection of *H. pylori* genes directly from gastric biopsy samples and from the corresponding *H. pylori* isolates, specimens from 31 patients were subjected to PCR with *ureC*- and *cagA*-targeting primers. All 31 biopsy specimens and the corresponding *H. pylori* isolates were positive in the *ureC* PCR. *H. pylori* strains that were *cagA* positive also gave positive *cagA* PCR fragments with biopsy specimens from the same patients. All *ureC* PCR-positive patients were examined; biopsy specimens from 10 of 11 (91.7%) duodenal ulcer patients harbored *H. pylori cagA*-positive strains, whereas 19 of 26 (73%) of those from patients with chronic gastritis only were found to be *cagA* positive. These findings indicate first that *ureC* PCR is at least as sensitive as culture for diagnosing *H. pylori* infection and second that the presence of the *H. pylori cagA* gene can also be detected directly in biopsy specimens by PCR amplification.**

The bacterium *Helicobacter pylori* is a fastidious, microaerophilic spiral gram-negative microorganism which plays a significant role in the pathogenesis of chronic gastritis, peptic ulcer, and, with mounting evidence from recent data, gastric cancer (4).

Its worldwide distribution and high level of prevalence and the importance of associated pathologies make the elimination of *H. pylori* a very useful approach to treating and controlling these gastroduodenal diseases, since its eradication results in a marked reduction in the rate of recurrence of duodenal and gastric ulcer (16).

Methods that accurately detect *H. pylori* infection in dyspeptic patients are therefore of major importance. Direct demonstration of *H. pylori* in gastric biopsy specimens is possible through the use of culture, histological examination with several stains, and assays for rapid urease activity. Culture, although labor-intensive because of the fastidious nature of the organism, is a very sensitive detection method with the advantages of specifically detecting *H. pylori* and making strains available for susceptibility testing. All these endoscopy-based methods require gastric biopsy specimens and are thus classified as invasive methods (14).

Recently, assays based on PCR technology were employed to detect the presence of *H. pylori* DNA by using several gene targets. Although the PCR technique looks very promising, in many of the reported studies either a limited number of specimens were analyzed or gastric biopsy specimens from selected unconsecutive patients were tested (3, 5, 15, 17, 19, 25, 27).

The cytotoxin-associated antigen (*cagA*) gene is an *H. pylori* gene that codes for a 96- to 138-kDa protein that is associated with the production of vacuolating toxin (6, 24). The presence of *H. pylori cagA*-positive strains and the serological response to the CagA protein are associated with peptic ulcer disease (7–9). Recently, a higher level of prevalence of anti-CagA antibodies in sera from patients with gastric cancer has also been reported (11).

The present study aimed (i) to comparatively evaluate PCR and other diagnostic methods (culture, rapid-urease test, and Giemsa staining of histological sections) for detection of *H. pylori* in biopsy specimens from 104 consecutive dyspeptic patients and (ii) to evaluate the detection of a putative virulence marker of *H. pylori*, the *cagA* gene, by PCR in biopsy specimens as well as in corresponding *H. pylori* isolates.

## MATERIALS AND METHODS

**Bacterial strains.** Thirty-five *H. pylori* isolates were used in this study: 3 *cagA*-positive reference strains (NCTC 11637, ATCC 49503, and ATCC 53726), a *cagA*-negative reference strain (Tx30A), and 31 clinical isolates from patients. Also tested were *Helicobacter mustelae* NCTC 12031, *Helicobacter fennelliae* NCTC 11612, *Helicobacter cinaedi* LMG 7543, *Campylobacter fetus fetus* ATCC 27374, *Campylobacter jejuni jejuni* NCTC 11351, *Campylobacter jejuni doylei* NCTC 11951, *Campylobacter coli* NCTC 11366, *Campylobacter lari* NCTC 11352, *C. sputorum* biovar *fecalis* NCTC 11415, the urease-positive strain *C. lari* J116, and clinical isolates (St. Pieters University Hospital, Brussels, Belgium) of vari-

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TABLE 1. Primer sequences, primer annealing positions, and expected lengths of amplified DNA products

| Primer | Sequence | Annealing position[a] | Product length (bp) |
|---|---|---|---|
| *ureC* gene | | | |
| 93275 | 5′ AAG CTT TTA GGG GTG TTA GGG GTT T 3′ | 1293–1318 | 294 |
| 93276 | 5′ AAG CTT ACT TTC TAA CAC TAA CGC 3′ | 1587–1563 | 294 |
| *cagA* gene | | | |
| 93089 | 5′ AAT ACA CCA ACG CCT CCA AG 3′ | 2593–2612 | 400 |
| 93261 | 5′ TTG TTG CCG CTT TTG CTC TC 3′ | 2992–2973 | 400 |

[a] The positions of the *ureC*-specific primers were reported by Labigne et al. (18); the positions of the *cagA*-specific primers in the sequence were reported by Tummuru et al. (24).

ous urease-positive bacterial species belonging to the family *Enterobacteriaceae* (five *Proteus mirabilis* isolates, seven *Klebsiella pneumoniae* isolates, and one *K. oxytoca* isolate), as well as a few nonfermentative gram-negative bacilli (two *Pseudomonas aeruginosa* isolates), which were all identified by routine methods (2). The strains were tested by PCR to assess the specificities of the primers. All strains were stored at −70°C in whole horse blood before being assayed by PCR.

**Patients.** One hundred four consecutive untreated patients undergoing upper gastroduodenal endoscopy from 1 October to 30 November 1993 at the Nouvelle Clinique de la Basilique (Brussels, Belgium) were included in the study. The population consisted of 40 males and 64 females with a mean age of 55.3 years (range, 19 to 93 years).

**Endoscopy and biopsy sampling.** At least five antrum and four corpus biopsy specimens were collected from each patient for the rapid-urease test (one antrum biopsy specimen), histology (three or more antrum and two or more corpus biopsy specimens), and culture (one each antrum and corpus biopsy specimens). Biopsy specimens for culture examination were dipped with sterile cotton in a semisolid agar transport medium (Portagerm; bioMérieux, Marcy l'Etoile, France). An additional antrum biopsy specimen was obtained for PCR analysis. Biopsy specimens for culture and PCR were frozen at −70°C until processing.

The endoscopic apparatus (Olympus GIF-IT or Fujinon UGIF-P2), including all channels, and biopsy forceps were carefully cleaned and disinfected by immersion in a 2% glutaraldehyde (Cidex; Surgikos Ltd., Livingstone, Scotland) solution for 15 min, rinsed in water, and dried after use with each patient and at the end of each endoscopic session.

**Culture.** Gastric biopsy specimens were ground at 10,000 rpm for 15 s with an electric tissue homogenizer (Ultraturrax, Iena, Germany) and then inoculated onto selective Columbia blood agar and incubated under microaerophilic (5% $O_2$, 10% $CO_2$, and 85% $N_2$) conditions at 37°C for up to 7 days (12). Organisms were identified as *H. pylori* on the basis of morphology in Gram stain and by oxidase, catalase, and rapid-urease tests (2).

**Histological examination.** Paraffin-embedded tissue sections were stained with hematoxylin and eosin to grade the severity of gastritis and with Giemsa stain to detect *H. pylori*. The classification and grading of gastritis were made in accordance with the Sydney system (21).

**Rapid-urease test.** One antrum biopsy specimen was introduced with a sterile needle into a semisolid 2% urea agar and incubated at room temperature. Results were recorded up to 4 h after inoculation (12).

**Case definition.** A patient was classified as *H. pylori* positive on the basis of a positive culture result or both a positive rapid-urease test and a positive histological examination.

**Preparation of samples for PCR amplification.** Genomic DNAs were extracted from all strains by the guanidium thiocyanate method (20). The extracted DNAs were dissolved in water, and solutions containing 25 μg of DNA per ml were prepared and used throughout the study. DNAs from biopsy specimens were prepared as follows. Two hundred microliters of 0.5% *N*-acetyl-L-cysteine (Sigma, St. Louis, Mo.) solution was added to the biopsy specimen, which was vortexed for 5 min and held at room temperature for 5 additional min. Tubes were centrifuged for 15 min at 17,000 × *g*. After the supernatants were discarded, biopsy specimens were resuspended in 100 μl of distilled water and an equal volume of 50 mM NaOH was then added. The tubes were vortexed vigorously and spun down briefly. The mixture was overlaid with 200 μl of light mineral oil (Sigma). The tubes were heated to 100°C for 10 to 15 min in a water bath. After cooling, 16 μl of 1 M Tris-HCl, pH 7.5, was added underneath the oil layer. Samples were stored at 4°C until tested.

**Primers.** All primers were synthesized on a model 394 DNA/RNA synthesizer (Applied Biosystems, Foster City, Calif.) by the phosphoramidite coupling method and purified as described by the manufacturer. Primer sequences, primer annealing position, and expected lengths of amplified DNA products are shown in Table 1. Primers 93275 and 93276, which target the *ureC* gene, are derived from the sequenced urease genes (EMBL accession no. X57132 and GenBank accession no. M60398) (3, 18). Primers 93089 and 93261 were selected from consensus regions of the two available *cagA* gene sequences (GenBank accession no. L11714 and EMBL accession no. X70039) (6, 24).

**PCR conditions.** Reactions were performed in a 50-μl volume in a Techne PHC 3 thermocycler (New Brunswick Scientific, Edison, N.J.). Reaction mixtures containing 0.4 μM each primer, 0.2 mM each deoxynucleoside triphosphate (dATP, dGTP, dTTP, and dCTP) (Boehringer Mannheim GmbH, Mannheim, Germany), and reaction buffer (100 mM Tris-HCl [pH 8.3], 500 mM KCl, 15 mM $MgCl_2$, and 0.01% [wt/vol] gelatin) were overlaid with 1 drop of light mineral oil (Sigma) before addition of 10 μl of test sample. The mixture was denatured at 94°C for 10 min and cooled on ice, and 2.5 U of *Taq* DNA polymerase (Boehringer) was added prior to 35 amplification cycles. For *ureC* amplification, cycles comprising a 2-min denaturing step at 94°C, a 2-min annealing step at 55°C, and a 2-min elongation step at 72°C were used (18). The *cagA* amplifications were done with cycles of 1 min of denaturation at 94°C, 1 min of annealing at 59°C, and 1 min of elongation at 72°C. After 35 cycles, a final cycle comprising a 1-min step at the specified annealing temperature and a 10-min elongation step at 72°C was performed. DNA from *H. pylori* ATCC 53726 and a tube containing water in place of DNA were assayed in each PCR run as positive and negative controls, respectively, of the reaction. To avoid cross-contaminations, mixture preparation, sample handling, PCR amplification, and gel detection of amplified material were done in separate rooms.

**Detection and analysis of amplified DNA products.** Ten-microliter amounts of each PCR mixture were subjected to gel electrophoresis (2% agarose) and ethidium bromide staining for the detection of amplified DNA products. The specificities of the amplified DNA fragments were assessed by restriction endonuclease digestion for 1 h at 37°C in the buffer recommended by the manufacturer. All enzymes and buffers were from Gibco BRL Life Technology Ltd. (Renfrewshire, Scotland). The amplified *ureC* products were digested with *Sph*I (restriction fragments of 168 and 126 bp) and *Nla*III (restriction fragments of 126, 90, and 78 bp). *Spe*I, which generates fragments of 274 and 126 bp, and *Hin*dIII, which generates 195-, 159-, and 46-bp fragments, were used to digest the amplified *cagA* products. Digested samples were analyzed by electrophoresis with 3% Nusieve–1% SeaKem gels (FMC Bioproducts, Rockland, Maine) and ethidium bromide staining (22).

## RESULTS

**Sensitivity and specificity of PCR assays with bacterial isolates.** Tenfold serial dilutions of DNA from *H. pylori* ATCC 53276, starting at a concentration of 36.0 ng/μl, were subjected to amplification by both primer pairs in order to assess the sensitivity of PCR detection. Both PCR assays were able to detect at least 3.6 fg of bacterial DNA, which corresponds to approximately two *H. pylori* genomes (23). The specificities of PCRs with primers targeting *ureC* and *cagA* (herein referred to as *ureC* and *cagA* PCRs, respectively) were determined by testing 25 bacterial strains from closely related genera and from various urease-positive bacterial species which may occasionally occur as contaminants in gastric biopsy specimens.

*ureC* PCR amplified the expected 294-bp fragment from all *H. pylori* reference and clinical strains, but none of the other bacterial species tested showed any amplicons (Fig. 1A).

Amplification of DNA from *H. pylori* ATCC 53726, ATCC 49503, and NCTC 11367 revealed the expected 400-bp PCR fragment when the two specific oligonucleotide primers targeting the *cagA* gene were used (Fig. 1B). No other bacterial species tested showed any product in *cagA* PCR. Digestion of the *ureC* PCR-amplified fragment with *Sph*I and *Nla*III gave the expected restriction endonuclease patterns, as did digestion of the 400-bp amplified fragment from *cagA* PCR with *Spe*I and *Hin*dIII (data not shown).

**Diagnostic assays.** Thirty-eight (36.5%) of the biopsy spec-

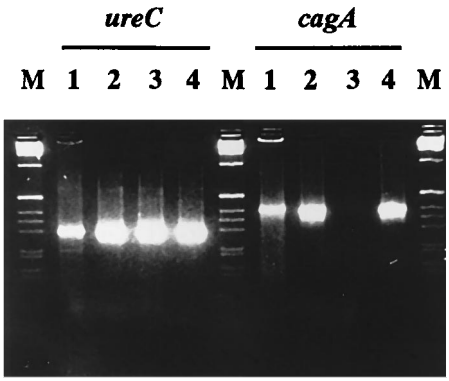


FIG. 1. PCR amplification, performed with *ureC* and *cagA* primer pairs, of a biopsy specimen (lane 1), the corresponding *H. pylori* isolate (lane 2), *H. pylori* Tx30A (lane 3), and *H. pylori* ATCC 53726 (lane 4). Lanes M, molecular size standards (1-kb DNA ladder; Gibco BRL).

TABLE 3. Detection of *H. pylori* by four invasive diagnostic techniques

| Result for diagnostic technique[a] | | | | No. of patients | |
|---|---|---|---|---|---|
| *ureC* PCR | Culture | Rapid-urease test | Histology | With chronic gastritis[b] | Total |
| + | + | + | + | 33 (28) | 33 |
| + | + | − | + | 1 (1) | 1 |
| + | + | + | − | 1 (0) | 1 |
| + | + | − | − | 3 (2) | 3 |
| + | − | + | − | 1 (0) | 1 |
| + | − | − | − | 0 (0) | 1 |
| − | − | − | + | 1 (1) | 1 |
| − | − | + | − | 1 (1) | 1 |
| − | − | − | − | 16 (0) | 62 |

[a] Biopsy specimens from 104 consecutive dyspeptic patients were tested.
[b] Numbers in parentheses are numbers of patients with chronic active gastritis. Gastritis was defined according to reference 21. A total of 57 patients had chronic gastritis; 33 of these had chronic active gastritis.

imens yielded cultures positive for *H. pylori*. The frequency of *H. pylori* positivity by culture in relation to the clinical diagnosis as determined by endoscopy is shown in Table 2.

The rapid-urease test was positive for 36 of the 104 (34.6%) patients' biopsy specimens. In one case, the urease test was the only positive test, while for another patient, *ureC* PCR was also positive. *H. pylori* organisms were detected by Giemsa staining in 35 (33.7%) of the specimens, being the only positive test on one occasion. The specific *ureC* gene fragment was amplified in biopsy specimens from 40 (38.5%) patients. The biopsy specimens of all culture-positive patients were positive by *ureC* PCR. Furthermore, biopsy specimens from two culture-negative patients yielded the expected 294-bp *ureC* fragment following PCR amplification (Table 3).

The comparative results obtained by all the different methods are reported in Table 3. Culture was taken as the "gold standard" for *H. pylori* detection with biopsy specimens, in view of its high specificity and because it detected the largest number (38 patients) of *H. pylori*-positive patients among the routine diagnostic assays. The sensitivity, specificity, and positive and negative predictive values for *ureC* PCR were, respectively, 100, 97, 95, and 100%; for the rapid-urease test they were 89.5, 97, 94.4, and 94.1%; and for histology they were 89.5, 98.5, 97.1, and 94.2%.

**Detection of the *cagA* gene.** Of the 40 *ureC* PCR-positive biopsy specimens, 32 (including those of the two culture-negative patients) also gave amplified DNA fragments in *cagA* PCR. All but one biopsy specimen from peptic ulcer patients were infected with *H. pylori cagA*-positive strains. The biopsy specimens from 19 (73%) of the patients with gastritis only also had *H. pylori cagA*-positive strains, as did the biopsy specimens from the gastric cancer patient. The comparison of the frequency of *cagA*-positive patients between the groups of peptic ulcer and gastritis-only patients did not reveal any statistically significant difference (two-tailed Fisher's exact test; $P = 0.393$).

**Correlation between PCR amplifications with biopsy specimens and with the corresponding *H. pylori* isolates.** In order to assess the likelihood of amplification of *H. pylori* genes directly from biopsy specimens, 31 available *H. pylori* isolates and their respective biopsy specimens were subjected in parallel to *ureC* and *cagA* PCRs. There was an excellent correlation between the results of PCR for the *H. pylori* isolates and the direct amplification of the *ureC* and *cagA* fragments from the corresponding biopsy specimens. The *ureC* fragment was amplified in all 31 *H. pylori* isolates as well as in their corresponding biopsy specimens. Twenty-five of the 31 isolates and the corresponding biopsy specimens showed the 400-bp *cagA* product. On the other hand, the biopsy specimens corresponding to all six *cagA* PCR-negative isolates were also negative (Fig. 1).

TABLE 2. *H. pylori* infection status and associated gastroduodenal pathology in patients[a]

| Culture result | No. of patients with gastroduodenal pathology | | | | | Total |
|---|---|---|---|---|---|---|
| | Normal[b] | Gastritis | Duodenal ulcer | Gastric ulcer | Other | |
| Positive | 0 | 25 | 11 | 1 | 1[c] | 38 |
| Negative | 62 | 1 | 0 | 2 | 1[d] | 66 |
| Total | 62 | 26 | 11 | 3 | 2 | 104 |

[a] Group of 104 consecutive dyspeptic patients undergoing upper gastroduodenal endoscopy.
[b] Endoscopically normal gastric mucosa.
[c] Gastric cancer patient.
[d] Esophageal cancer patient.

## DISCUSSION

In this study, we have investigated a group of consecutive dyspeptic patients by endoscopy and gastric biopsies for *H. pylori* infection. We found that *ureC* PCR was at least as sensitive as culture for detecting *H. pylori* infection in patients. All culture-positive patients were also *ureC* PCR positive. The rapid-urease test and histological examinations of biopsy specimens detected *H. pylori* infection in slightly fewer patients than did culture but they still yielded good specificity, although they gave some false-positive results (two and one, respectively). Overall, our results are in good agreement with other recent data for comparisons of different routine tests for *H. pylori* in which Giemsa staining of histological sections (26) or the agreement among all the tests was taken as the gold standard (13). In both studies PCR assays detected *H. pylori* infection in a higher percentage of patients.

*ureC* and *cagA* PCRs were able to detect up to the equivalent of two *H. pylori* genomes in a sample, which is in the same range of detection as that described for other PCR assays for *H. pylori* (5, 15, 25). However, our assay was more sensitive than the one reported in which the same *ureC* primers but shorter steps in each cycle (1 min) were used (3).

The apparent lack of specificity of *ureC* PCR may actually relate to the use of culture as the gold standard for comparison. In our study, two patients who each had culture-negative spec-

imens had biopsy specimens which yielded amplifiable *ureC* PCR products. For one of the patients, the rapid-urease test was also found to be positive and the histological examination revealed chronic gastritis; the patient had a history of duodenal ulcer and of *H. pylori* infection. At the time that endoscopy was performed, the patient had received a 7-day course of erythromycin for an upper respiratory tract infection, and this could probably have resulted in the suppression of the organisms to levels undetectable by culture and histology. The second patient had a normal gastric mucosa at histology, and the positive PCR result could possibly reflect a recent *H. pylori* infection or a cross-contamination in the laboratory or perhaps from the endoscopic apparatus.

The specificity of the PCR assay with the *ureC* primers was once more verified (3) since *ureC* amplifications were obtained only with *H. pylori*, while none of the other urease-positive or related bacteria that were tested gave the expected amplified DNA products. The results of PCR amplification directly from biopsy specimens and from the *H. pylori* strains subsequently isolated from them were perfectly correlated. No false-positive or false-negative results were found for biopsy specimens amplified by the *ureC* or *cagA* primers, suggesting the absence of inhibitory substances in gastric biopsy specimens.

The CagA protein is a surface immunodominant antigen (1) with a variable molecular mass of 96 to 138 kDa depending on the number of intragenic repeats (6, 24). Although its function has not yet been established, the CagA protein is present in 60 to 80% of *H. pylori* strains, and it is found in close association with the production of vacuolating cytotoxin (7, 9). Its frequency in *H. pylori* strains isolated from peptic ulcer patients is greater than in those isolated from patients with gastritis only (8). Anti-CagA antibodies in serum (immunoglobulin G) and in supernatants from gastric biopsy cultures (immunoglobulins G and A) have shown that CagA is produced in vivo (7, 10, 11) and that this protein is also detected more frequently in peptic ulcer patients than in gastritis-only patients (7, 8, 10). There is also a high percentage of detection of anti-CagA antibodies in gastric cancer patients (11).

In this study, the 400-bp *cagA* amplified DNA fragment was found in the biopsy specimens from 32 (80.0%) of the 40 *ureC* PCR-positive patients. Similar frequencies of *cagA* detection were found for a selected series from the same population (8) and in other serological studies of CagA (1, 28). Although all *H. pylori*-positive peptic ulcer patients except one were infected with *H. pylori cagA*-positive strains, we could not demonstrate any significant difference between the frequency of *cagA*-positive strains among infected patients with duodenal or gastric ulcer and that among subjects with gastritis only. However, this analysis could probably have been impaired by the small number (11 patients) of peptic ulcer patients evaluated.

In conclusion, we found that the PCR assay targeting the *ureC* gene in biopsy specimens proved at least as sensitive as culture for detecting *H. pylori* infection in patients. In addition, although we do not yet have a comprehensive survey of post-treatment patients, we think that the *ureC* PCR could constitute a very useful diagnostic tool for treatment follow-up, at which time the number of bacteria in the gastric mucosa is usually small and may go undetected by culture or by other diagnostic methods. The amplification of *H. pylori* genes directly from biopsy specimens matched closely their detection in the corresponding *H. pylori* isolates. This study also showed that PCR has a potential value for studying *cagA* and possibly other virulence genes directly from biopsy specimens by PCR, allowing rapid determination of whether patients are at high risk for peptic ulcer.

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