

## Magnetic Immuno-PCR Assay with Inhibitor Removal for Direct Detection of *Helicobacter pylori* in Human Feces

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**A PCR protocol was developed to detect *Helicobacter pylori* in human stool specimens. This protocol was based on the association of a magnetic immuno-PCR assay with a technique to remove inhibitors (agarose-embedded DNA preparation). Of the 47 *H. pylori*-positive and 57 *H. pylori*-negative patients included in this study, 38 were positive and 66 were negative by this new protocol. The sensitivity, specificity, and predictive values for a positive or a negative result were 80.9% (95% confidence interval [CI], 66.3 to 90.4), 100% (95% CI, 92.1 to 100), 100% (95% CI, 88.6 to 100), and 86.4% (95% CI, 75.2 to 93.2), respectively.**

The high clinical relevance of *Helicobacter pylori* infection has stimulated the development of numerous diagnostic methods. These methods can be classified as invasive, (i.e., those that require an endoscopy to detect *H. pylori* directly in gastric biopsy specimens) or noninvasive (i.e., based on the study of various samples (serum, breath air, urine, etc.)), which indirectly indicate the presence of *H. pylori*.

Stool specimens constitute a sample of easy, noninvasive access and consequently of high potential interest for the development of a direct method of *H. pylori* detection. PCR is a powerful technique for the detection of target DNA in various clinical specimens, but its application to stool specimens has been limited due to the presence of substances inhibiting the reaction. Numerous attempts to detect *H. pylori* by PCR in stool samples have been made, with controversial results (5, 12, 17; N. P. Mapstone, F. A. Lewis, D. S. Tompkins, D. A. F. Lynch, A. T. R. Axon, M. F. Dixon, and P. Quirke, Letter, Lancet **341**:447, 1993).

Difficulty in eliminating PCR inhibitors from stool specimens has been extensively reported (4, 5, 8, 12). Some authors have claimed to be successful, but usually only after applying complex procedures that are difficult to implement routinely.

The aim of this study was to develop a PCR protocol to detect *H. pylori* in human stool specimens that is simple enough to be used routinely. For this purpose, we associated a magnetic immuno-PCR assay (MIPA) with a technique to remove inhibitors that was recently described (11).

**Clinical samples.** One hundred four consecutive, untreated, dyspeptic patients (69 male, 35 female; mean age, 50 years; range, 17 to 87 years) who were consulting gastroenterologists for dyspepsia in Bordeaux, France, were included in the study. Exclusion criteria were as follows: *H. pylori* eradication treatment in the previous 6 months; consumption of antibiotics in the previous month; or consumption of antisecretory drugs, bismuth salts, or sucralfate in the previous 2 weeks. A history of coagulopathy or other disorders that are contraindications

for endoscopy and/or biopsy sampling was also a reason for exclusion. For each patient, biopsies were taken for culture, histological examination, urease test (UT), and PCR. A urea breath test (UBT) and a serological test were also performed as previously described (9).

A patient was classified as being *H. pylori* positive on the basis of (i) a positive culture; or, in the case of a negative culture, (ii) both a positive histological examination and a positive UT (CLOtest; TriMed Specialities, Osborne Park, Western Australia); or (iii) positive noninvasive tests (serology and UBT) and biopsy PCR. Stool specimens were collected within 1 week of the time of endoscopy in sterile containers and kept at  $-80^{\circ}\text{C}$  until analysis.

**Immunomagnetic separation of *H. pylori* from human feces and DNA extraction.** Stool specimens were suspended at 1.5:5 (wt/vol) for solid and semisolid samples and 1.5:5 (vol/vol) for liquid in phosphate-buffered saline and incubated overnight, under agitation at room temperature. The suspension was then filtered through three layers of cotton gauze and used for immunomagnetic separation of *H. pylori*. Briefly, magnetic uncoated beads (Dynabeads M-450; DYNAL, Oslo, Norway) were coated with rabbit anti-*H. pylori* immunoglobulin (Dako, Glostrup, Denmark) at a concentration of 5  $\mu\text{g}$  of antibody for  $10^7$  Dynabeads according to the procedure recommended by DYNAL. A 60- $\mu\text{l}$  volume of coated Dynabeads was mixed with 1 ml of fecal suspension and incubated at  $4^{\circ}\text{C}$  with continuous shaking for 2 h. The coated Dynabeads were recovered by magnetic force with a Dynalmagnet and then suspended in the lysis buffer of the QIAamp tissue kit (Qiagen, Hilden, Germany) for DNA extraction (9). Twenty microliters of a proteinase solution (20 mg/ml) was then added, followed by incubation at  $56^{\circ}\text{C}$  for 2 h. A second buffer provided in the kit was added, and the sample was incubated at  $70^{\circ}\text{C}$  for 10 min. Next, 200  $\mu\text{l}$  of ethanol was added, and the suspension was loaded on the QIAamp spin column followed by a centrifugation at  $6,000 \times g$  for 1 min. The QIAamp spin column was placed in a 2-ml collection microtube, and the tube containing the filtrate was discarded. The column material was washed twice (250  $\mu\text{l}$  each) with the first washing buffer and twice (250  $\mu\text{l}$  each) with the second washing buffer provided in the kit. Fi-

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TABLE 1. Diagnostic test results for the five patients for whom the results of invasive tests for *Helicobacter pylori* diagnosis were doubtful

Patient	Result by <sup>a</sup> :										
	Histology		Urease test		Culture		PCR with biopsy		UBT	Serology	PCR with feces
	Antrum	Fundus	Antrum	Fundus	Antrum	Fundus	Antrum	Fundus			
1	-	+	-	ND	-	ND	+	ND	+	+	+
2	-	+	-	ND	-	ND	+	ND	+	+	+
3	-	-	+	ND	-	ND	-	ND	-	-	-
4	-	-	+	ND	-	ND	-	ND	-	-	-
5	+	+	-	ND	-	ND	-	ND	-	-	-

<sup>a</sup> +, positive result; -, negative result; ND, not determined.

nally, the DNA was eluted with 100 µl of distilled water preheated to 70°C (2 × 50 µl).

**PCR.** PCR was performed as described previously (7). Briefly, reactions were carried out in a volume of 50 µl with a combination of 10× PCR buffer [670 mM Tris-HCl (pH 8.8), 160 mM (NH<sub>4</sub>)SO<sub>2</sub>, 0.1% Tween]; 1.5 mM MgCl<sub>2</sub>; 200 µM (each) nucleotides dATP, dTTP, dGTP, and dCTP; and 3 U of *Taq* polymerase (Eurobio, Les Ulis, France), with 5 µl of purified sample and with specific primers (HPU1 and HPU2) for the *ureA* gene of *H. pylori* (1) at 0.4 µM each. PCR consisted of 35 cycles of 1 min at 94°C, 1 min at 45°C, and 1 min at 72°C with a first cycle of 5 min at 95°C and a final cycle of 5 min at 72°C. Bands were visualized on a 1% agarose gel stained with ethidium bromide. The size of the PCR product obtained when using the HPU1 and HPU2 primers was 411 bp. In each experiment, a positive control consisting of 50 ng of reference strain DNA and a negative control (distilled water) were performed.

**Removal of PCR inhibitors.** The PCR inhibitors present in stool samples were removed by using an agarose-embedded DNA preparation described previously (9, 11). Briefly, 1 vol-

ume of 1.6% melted agarose was added to each DNA aliquot; these were then poured into molds (Disposable Plug Molds; 1.5 by 10 by 5 mm; Bio-Rad, Richmond, Calif.) until solidification. Agarose blocks were removed from molds and washed in Tris-EDTA (10 ml per block) overnight with gentle shaking. A second wash with 5 ml of distilled water was performed (2 h with gentle shaking). PCR was subsequently carried out with DNA-containing agarose slices as templates.

**Statistics.** Standard methods were used to calculate sensitivity, specificity, predictive values of positive and negative results, and the 95% confidence intervals (CI) of these values.

**Ethics.** This study received the approval of the Ethics Committee of Bordeaux. The informed consent of the patients was also obtained.

According to the case definition, 47 of the 104 patients tested were *H. pylori* positive, and 57 were negative. The five patients for which doubtful results were found based on invasive tests were evaluated according to the results of noninvasive tests and biopsy PCR (Table 1). Two were considered *H. pylori* positive, and three were considered *H. pylori* negative.

According to this new PCR protocol to detect *H. pylori* in

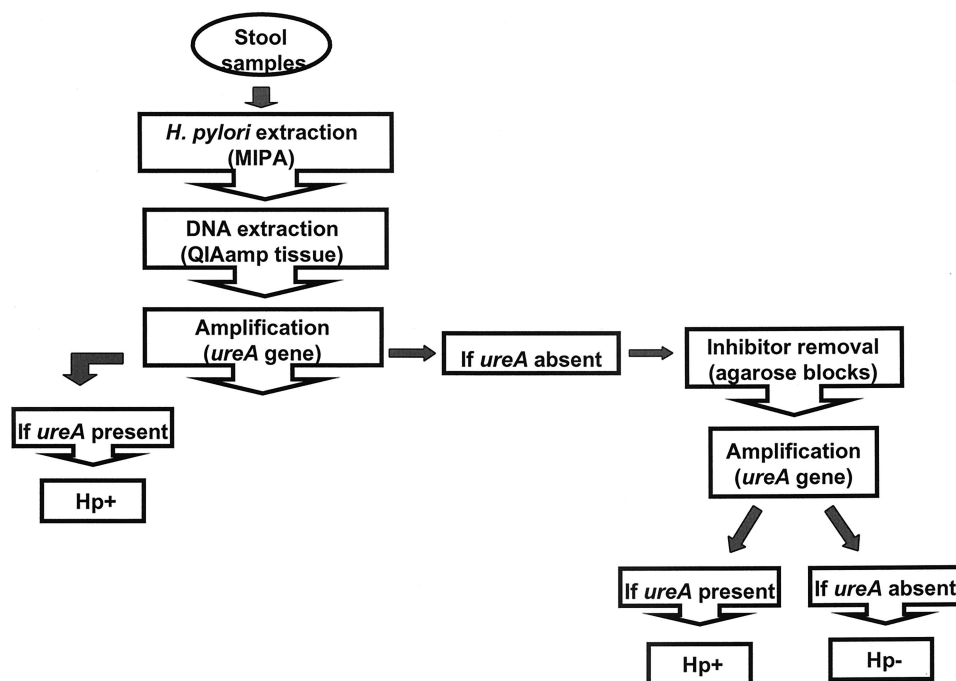


FIG. 1. Stool sample processing method used in this study.

human feces (Fig. 1), 38 of the 47 *H. pylori*-positive patients were *H. pylori* positive (giving 9 false negatives), and all 57 *H. pylori*-negative patients were also *H. pylori* negative with the new protocol (no false positive). The sensitivity, specificity, and predictive values for a positive or a negative result were 80.9% (95% CI, 66.3 to 90.4), 100% (92.1 to 100), 100% (88.6 to 100), and 86.4% (75.2 to 93.2), respectively. Most of the cases ( $n = 32$ ) were detected before agarose blocks were performed. However, six additional cases were detected with this method. The overall correlation between biopsy PCR and stool PCR was good (90 of 104). There were 11 cases positive only by biopsy PCR, and there were 3 cases positive only by stool PCR.

Detection of *H. pylori* from stool specimens as a diagnostic tool for detecting *H. pylori* infection is attractive because it is noninvasive. However, *H. pylori* has never been reliably detected from stools (3). The first reason is probably the lack of viability of these bacteria in an environment of competing flora under normal conditions in the bowel. In addition, selective culture media for the isolation of *H. pylori* are frequently contaminated with other, faster-growing gram-negative bacteria. The only positive attempts reported used feces obtained from patients experiencing spontaneous (15) or induced diarrhea (13).

Similarly, detection of *H. pylori* in stool specimens by using PCR after standard extraction methods has proved to be difficult (5, 12, 17; Mapstone et al., Letter), producing erratic results due to a variety of fecal inhibitors, such as acid polysaccharides, metabolic products, and large amounts of irrelevant DNA (8).

In this study, we developed a new PCR protocol to detect *H. pylori* in human feces. For this purpose, a MIPA was associated with an agarose-embedded DNA preparation to remove inhibitors (9). Successful amplification and specific detection of *H. pylori* DNA directly from stool samples in most of the infected patients indicate that MIPA, in association with the agarose-embedded DNA block method, is a promising procedure to detect *H. pylori* in stool specimens, but requires further development before it will be suitable for routine clinical use, because the sensitivity obtained is not sufficient. However, it is easier to apply than previously published methods (4, 5; Mapstone et al., Letter), avoiding the use of nested PCR. While nested PCR is supposed to increase sensitivity, it also has some limits, especially in routine use. The high risk of contamination makes it difficult to implement, and for this reason, such a procedure has not been recommended in the past (2).

Another stool test, based on the detection of *H. pylori* antigens to diagnose infection, is also currently available (16). However, despite the fact that this *H. pylori* antigen stool assay

is reliable and easy to perform, it only indicates the presence or absence of *H. pylori* based on the detection of antigens the exact character of which is unknown. In contrast, PCR can provide additional information, principally related to the presence of pathogenicity factors (*cagA* or *vacA* alleles) or susceptibility to macrolides, antibiotics commonly used to treat this infection (6, 14). This subject will be of great interest for future investigations.

#### REFERENCES

1. Clayton, C. L., H. Kleanthous, P. J. Coates, D. D. Morgan, and S. Tabaqchali. 1992. Sensitive detection of *Helicobacter pylori* by using polymerase chain reaction. *J. Clin. Microbiol.* **30**:192-200.
2. European *Helicobacter pylori* Study Group. 1997. Guidelines for clinical trials in *Helicobacter pylori* infection. *Gut* **41**(Suppl. 2):8-13.
3. Kelly, S. M., M. C. L. Pitcher, S. M. Farmery, and G. R. Gibson. 1994. Isolation of *Helicobacter pylori* from feces of patients with dyspepsia in the United Kingdom. *Gastroenterology* **107**:1671-1674.
4. Lantz, P.-G., M. Matsson, T. Wadström, and P. Rådström. 1997. Removal of PCR inhibitors from human faecal samples through the use of an aqueous two-phase system for sample preparation prior to PCR. *J. Microbiol. Methods* **28**:159-167.
5. Makristathis, A., E. Pasching, K. Schütze, M. Wimmer, M. L. Rotter, and A. M. Hirschl. 1998. Detection of *Helicobacter pylori* in stool specimens by PCR and antigen enzyme immunoassay. *J. Clin. Microbiol.* **36**:2772-2774.
6. Marais, A., L. Monteiro, A. Occhialini, M. Pina, H. Lamouliatte, and F. Mégraud. 1999. Direct detection of *Helicobacter pylori* resistance to macrolides by a polymerase chain reaction/DNA enzyme immunoassay in gastric biopsy specimens. *Gut* **44**:463-467.
7. Monteiro, L., C. Birac, and F. Mégraud. 1995. Detection of *Helicobacter pylori* in gastric biopsy by polymerase chain reaction, p. 112-120. In A. Lee and F. Mégraud (ed.), *Helicobacter pylori*: techniques for clinical diagnosis and basic research. W. B. Saunders Co., Ltd., London, England.
8. Monteiro, L., D. Bonnemaïson, A. Vekris, K. G. Petry, J. Bonnet, R. Vidal, J. Cabrita, and F. Mégraud. 1997. Complex polysaccharides as PCR inhibitors in feces: *Helicobacter pylori* model. *J. Clin. Microbiol.* **35**:995-998.
9. Monteiro, L., N. Gras, R. Vidal, J. Cabrita, and F. Mégraud. 2001. Detection of *Helicobacter pylori* DNA in human feces by PCR: DNA stability and removal of inhibitors. *J. Microbiol. Methods* **45**:87-94.
10. Monteiro, L., A. de Mascarel, A. M. Sarasqueta, B. Bergey, C. Barberis, P. Talby, D. Roux, L. Shouler, D. Goldfain, H. Lamouliatte, and F. Mégraud. 2001. Diagnosis of *Helicobacter pylori* infection: non-invasive methods compared to invasive methods and evaluation of two new tests. *Am. J. Gastroenterol.* **96**:353-358.
11. Moreira, D. 1998. Efficient removal of PCR inhibitors using agarose-embedded DNA preparations. *Nucleic Acids Res.* **26**:3309-3310.
12. Notarnicola, M., F. Russo, A. Cavallini, M. Bianco, E. Jirillo, S. Pece, C. Leoci, G. di Matteo, and A. di Leo. 1996. PCR identification of *Helicobacter pylori* DNA in faeces from patients with gastroduodenal pathology. *Med. Sci. Res.* **24**:785-787.
13. Parsonnet, J., H. Shmueli, and T. Haggerty. 1999. Fecal and oral shedding of *Helicobacter pylori* from healthy infected adults. *JAMA* **281**:2240-2245.
14. Pina, M., A. Occhialini, L. Monteiro, H.-P. Doermann, and F. Mégraud. 1998. Detection of point mutations associated with resistance of *Helicobacter pylori* to clarithromycin by hybridization in liquid phase. *J. Clin. Microbiol.* **36**:3285-3290.
15. Thomas, J. E., G. R. Gibson, M. K. Darboe, A. Dale, and L. T. Weaver. 1992. Isolation of *Helicobacter pylori* from human feces. *Lancet* **340**:1194-1195.
16. Vaira, D., P. Malfertheiner, F. Mégraud, and A. T. Axon. 1999. Diagnosis of *Helicobacter pylori* infection by HpSA test. *Lancet* **354**:1732.
17. van Zwet, A. A., J. C. Thijs, A. M. D. Kooistra-Smid, J. Schirm, and J. A. M. Snijder. 1994. Use of PCR with feces for detection of *Helicobacter pylori* infections in patients. *J. Clin. Microbiol.* **32**:1346-1348.