Detection of *Helicobacter pylori* in Gastric Biopsy Tissue by Polymerase Chain Reaction

J.T. Wang*, J.T. Lin, J.C. Sheu, J.C. Yang, D.S. Chen, T.H. Wang

To evaluate the sensitivity of a polymerase chain reaction (PCR) assay using nested primers in detecting *Helicobacter pylori*, gastric tissue biopsy specimens were collected on endoscopy from 17 patients with a duodenal ulcer. DNA was extracted by phenol/chloroform treatment or boiling in water, and then subjected to a nested PCR using two primer pairs from the urease gene of *Helicobacter pylori*. Fourteen of the 17 patients were positive for *Helicobacter pylori* using DNA samples extracted by either method. The PCR results correlated well with the results of an enzyme immunoassay to detect IgG antibody. However, there were two culture negative patients. The three PCR negative patients were both culture negative and serologically negative. DNA from 9 of the 14 patients was randomly selected and subjected to semiquantification by serial dilutions, and then PCR. The results showed that phenol/chloroform extraction yielded 10–1000 times more DNA than the boiling method. It is concluded that the PCR assay is a rapid and sensitive method for detecting *Helicobacter pylori*, and that phenol/chloroform extraction is superior to simple boiling in obtaining DNA samples for PCR.

Helicobacter pylori is a gram-negative bacterium associated with chronic gastritis and peptic ulcer disease of humans (1, 2). A number of methods have been adopted to diagnose infection with this organism (3–8). Several techniques used in molecular biology have been employed recently to detect *Helicobacter pylori*, including the use of radio-labelled and antigenically labelled chromosomal fragments, in situ hybridization, hybridization with an oligonucleotide complementary to the 16S rRNA sequence and the polymerase chain reaction (PCR) (9–12). Analysis by the PCR has been shown to be a rapid, sensitive and specific method for detection of *Helicobacter pylori* (12). For this assay, DNA can be extracted from gastric tissue biopsy material by two methods: traditional phenol/chloroform extraction and boiling of the tissue in water (13, 14). Although the boiling method is time-saving and more economical, the efficiency of the method compared to phenol/chloroform extraction has not been established. In this study, we determined the sensitivity of a PCR method in detecting *Helicobacter pylori* and compared the efficiency of the two DNA extraction methods.

Department of Internal Medicine, National Taiwan University Hospital, No. 1 Chang-Te St, Taipei, Taiwan 10016.

Materials and Methods. From March to May 1991, 17 patients diagnosed by endoscopy as having a duodenal ulcer were enrolled for the study. They all gave informed consent. Blood samples were obtained immediately after endoscopic examination, and were tested for IgG antibody to *Helicobacter pylori* with an enzyme immunoassay (Amrad, Australia). Biopsy specimens from each patient were taken from the antrum of the stomach with a new, unused biopsy forceps. For each patient, two specimens were sent immediately for culture and the other specimens were frozen immediately and kept at -80 °C until tested.

Two biopsy specimens from each patient were subjected to DNA extraction by two different methods. One specimen was minced, digested overnight in a solution containing 100 μg/ml proteinase K, 10 mM Tris-Cl (pH 8.0) 0.1 M EDTA and 0.5 % SDS, and then subjected to phenol/chloroform extraction using a single-tube method (Biotecx Laboratories, USA) according to the manufacturer's instructions. After alcohol precipitation, the DNA pellet was dissolved in 100 μl of water. The other specimen was minced and then boiled in 100 μl of water for 15 min. After centrifugation, the supernatant was removed and the precipitate discarded.

Ten μl of each DNA solution was subjected to a two-step nested PCR using two primer pairs from the urease gene of the *Helicobacter pylori* genome (15). The outer primer pair was 5' G CCA ATG GTA AAT TAG TTCC (nucleotide 304), 5' CTC CTT AAT TGT TTT TAC AT (nucleotide 714R) (13). The expected size of the PCR product was 411 bp. The PCR was carried out in a 50 μl mixture containing 10 mM tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01 % gelatin, 2.5 units of recombinant *Taq* DNA polymerase (Perkin-Elmer Cetus, USA), 200 μM dNTP, 0.6 μM of each outer primer and 5 μl of the

DNA mixture. A fast temperature cycle was performed as described previously to improve sensitivity (16). Briefly, 40 cycles of 96 °C for 30 seconds, 56 °C for 15 seconds and 74 °C for 30 seconds were performed, the temperature being maintained at 74 °C for 10 min at the end of the last cycle. The two 20-nucleotide sequences from the urease A gene used as the nested inner primers were 5' AGT TCC TGG TGA GTT GTT CT (nucleotide 318), 5' AGC GCC ATG AAA ACC ACG CT (nucleotide 678R) (15). The expected PCR product was 361 bp. The second round of the PCR was performed as described previously (17). Briefly, 1 µl of the reaction mixture was transferred to the second round reaction mixture containing 0.6 µM of each inner primer and the same buffer as in the first round. The temperature cycle of the second round PCR was also the same. Ten µl of the first and second round PCR products were electrophoresed on a 2 % agarose gel and then stained with ethidium bromide.

Samples of the same patient were tested in aliquots of the same reagents under the same reaction conditions. A DNA fragment containing the sequences of the urease gene was cloned and serially diluted. Samples with 1–1000 copies of the cloned DNA fragment were used to determine sensitivity. Culture negative gastric tissue biopsy specimens from healthy persons and reagents without DNA were used as negative controls in the PCR assay. For each round of the PCR, cloned DNA fragment with a volume of DNA supernatant extracted from the clinical samples was used as a control for false-negative reactions due to the presence of *Taq* inhibitory substrates. The measures suggested by Kwok and Higuchi (18) were applied to avoid contamination.

Results and Discussion. The sensitivity level of the first PCR round was 1000 copies of the cloned *Helicobacter pylori* DNA fragment, while in the second PCR round a single copy of the DNA fragment was detected (Figure 1). No false positive result was observed in the negative controls including the culture-negative gastric tissue from healthy persons. Using phenol/chloroform DNA extraction, ten patients were positive for *Helicobacter pylori* in the first PCR round, while only three were positive using the boiling method (Table 1). In the second PCR round, 14 of the 17 patients were positive for *Helicobacter pylori* using both extraction methods (Figure 2). PCR products of two patients were subjected to direct sequencing to confirm the specificity of the PCR. Both had a homology of approximately 98 % in 183 base pairs compared with the reported sequences (15) (Figure 3). All 14 PCR-positive patients were also seropositive for IgG antibody;

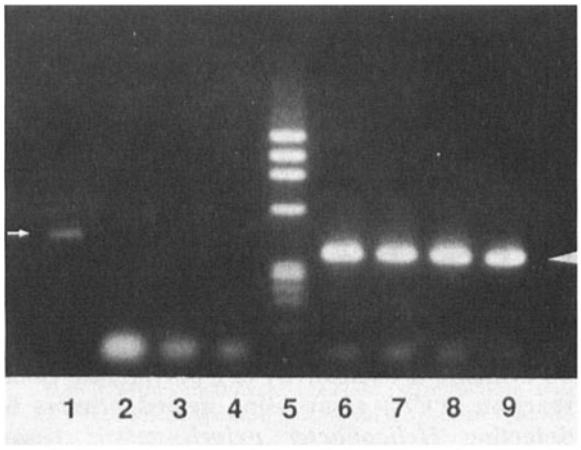

Figure 1: Sensitivity of PCR to detect *Helicobacter pylori*. Lanes 1–4: first PCR round products of 1000, 100, 10, 1 copies of *Helicobacter pylori* DNA fragment (1 copy of cloned DNA fragment = 10^{-3} fg); lane 5: φ x 174/*Hae*III; lanes 6–9: second PCR round products of 1000, 100, 10, 1 copies of *Helicobacter pylori* DNA fragment. Arrow: 411 base pairs; arrowhead: 361 base pairs.

Table 1: Results of tests to detect *Helicobacter pylori* in 17 patients with duodenal ulcer.

Patient no.	PCR		Serological test	Culture[c]
	1st round[a]	2nd round[b]		
1	+(+)	+	+	+
2	+(–)	+	+	+
3	+(–)	+	+	+
4	+(–)	+	+	+
5	+(–)	+	+	+
6	+(–)	+	+	–
7	–(–)	+	+	+
8	–(–)	+	+	–
9	–(–)	+	+	+
10	+(–)	+	+	ND
11	+(+)	+	+	ND
12	+(+)	+	+	ND
13	+(–)	+	+	ND
14	–(–)	+	+	ND
15	–(–)	–	–	–
16	–(–)	–	–	–
17	–(–)	–	–	–

[a] Results for phenol/chloroform extraction with results for the boiling method in brackets.
[b] Results of the second PCR round were the same for both extraction methods.
[c] ND = not done.

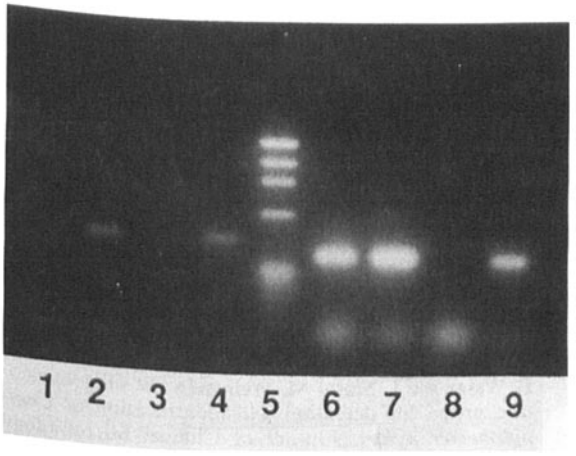

Figure 2: First and second PCR round results in clinical samples. Lane 1: first PCR round product in patient no. 2 using the boiling method for DNA extraction; lane 2: first PCR round product in patient no. 2 using the phenol/chloroform extraction method; lane 3: negative control; lane 4: positive control; lane 5: φ x 174/*Hae*III; lanes 6–9: second PCR round products of samples in lanes 1–4, respectively.

Table 2: Semiquantification of *Helicobacter pylori* DNA by the nested PCR. Numbers indicate maximal dilution positive in the nested PCR.

Patient no.	Boiling method	Phenol/chloroform method
1	10^3	$>10^4$
2	10^2	$>10^4$
3	10^3	$>10^4$
4	10^2	$>10^4$
5	10	$>10^4$
6	10	10^4
7	1	10^4
8	1	10^4
9	1	10^3

two were culture negative. All three PCR-negative patients were also negative for *Helicobacter pylori* on culture and in the serological test. Nine of the 14 PCR-positive DNA samples were selected and subjected to semiquantification by ten-fold serial dilution and nested PCR. Semiquantification of the DNA showed that the boiling method extracted about 10–1000 times less than phenol/chloroform extraction (Table 2).

The fact that in 14 of the 17 patients *Helicobacter pylori* was detected confirms the possibility of an active role of *Helicobacter pylori* in peptic ulcer disease. Because the clinical response in such cases is related to eradication of the bacteria with antibiotic therapy (19), a rapid, sensitive and specific test to detect *Helicobacter pylori* would be of great value in the management of these patients. Although currently there are various tests for the detection of *Helicobacter pylori*, such as the C-urea breath test, the urea hydrolysis test and staining techniques, they are either time-consuming or insensitive (3, 5, 8, 20–22). Serological tests are now widely used for both epidemiologic studies and diagnosis (4), and excellent correlation was shown between serological and PCR results in our study. However, serologic tests cannot be used to differentiate a current infection from past *Helicobacter pylori* infection (4, 23). *Helicobacter pylori* can be cultured from gastric tissue (3, 5, 21), but the organism is fastidious and grows slowly, usually requiring three to seven days of incubation (5, 24). Furthermore, culture was shown to be less sensitive than PCR in our study.

A previous study using the same outer primer pair and a single PCR round demonstrated high specificity of PCR in detecting *Helicobacter pylori* (13). To confirm the specificity of the primers and to analyze the heterogeneity of the bacterial genome, analysis of partial sequences of

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                    ⇓
Urease A 1 ---  AGACCGGTTCAAATCGGCTCACACTTCCATTTCTTTGAAGTGAATAGATGCTTAGACTTTG
Patient 1       ------------------------------------------------------------
Patient 12      ------------------------------------------------------------

Urease A 1      ACAGAGAAAAAACTTTCGGTAAACGCTTAGACATTGCGAGCGGGACAGCGGTAAGGTTTGA
Patient 1       ----------------------------C-------------------------------
Patient 12      ----------------C-------------------------------------------

Urease A 1      GCCTGGCGAAGAAAAATCCGTAGAGTTGATTGACATTGGCGGTAACAGAAGAATCTTTGGA
Patient 1       A----------------------A-------------------------------------
Patient 12      A----------------------A-------------------------------------
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Figure 3: Partial nucleotide sequences of PCR products of patients no. 1 and no. 12 compared with the reported urease A sequences. Urease A 1: the reported sequences (reference 15). Arrow: nucleotide 409. Both strains show 98 % homology in 183 nucleotides.

the PCR products of two patients in our series was done. The analysis revealed that the urease A gene is highly conserved in strains from different areas of the world. Therefore primers from this region are very useful for diagnostic PCR. The PCR assay with nested primers was shown to be sensitive in the detection of *Helicobacter pylori* DNA in clinical biopsy samples. The technique could thus be used to predict a relapse of infection after antibacterial therapy. The assay also has the potential to differentiate bacterial strains when a less conserved region of the genome is analyzed, once we know more about the genomic heterogeneity of the organism. Although in this study the boiling DNA extraction method was demonstrated to be less efficient than the phenol/chloroform DNA extraction method, the rate of positive results in subsequent nested PCR was the same for both methods in 17 patients. Therefore, nested PCR analysis of clinical biopsy samples with DNA extraction by the boiling method would seem to be a practical method. However, if the bacteria content in the stomach is low, such as when patients are receiving antibiotic treatment, the boiling method may yield false-negative results.

We conclude that the nested PCR is a sensitive and specific method for detecting *Helicobacter pylori* in gastric tissue. The efficiency of DNA extraction by the boiling method is low compared to the traditional phenol/chloroform method, but the boiling method is faster and less costly.

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References

1. **Buck GE:** *Campylobacter pylori* and gastroduodenal disease. Clinical Microbiology Reviews 1990, 3: 1–12.
2. **Graham DY:** *Campylobacter pylori* and peptic ulcer disease. Gastroenterology 1989, 96: 615–625.
3. **Coudron PE, Kirby DF:** Comparison of rapid urease tests, staining techniques, and growth on different solid media for detection of *Campylobacter pylori*. Journal of Clinical Microbiology 1989, 27: 1527–1530.
4. **Evans DJ, Evans DG, Graham DY, Klein PD:** A sensitive and specific serologic test for detection of *Campylobacter pylori* infections. Gastroenterology 1989, 96: 1004–1008.
5. **Goodwin CS, Blincow ED, Warren JR, Waters TE, Sanderson CR, Easton L:** Evaluation of cultural techniques for isolating *Campylobacter pylori* from endoscopic biopsies of gastric mucosa. Journal of Clinical Pathology 1985, 38: 1127–1131.
6. **Marshall BJ, Surveyor I:** Carbon-14 urea breath test for the diagnosis of *Campylobacter pylori* associated with gastritis. Journal of Nuclear Medicine 1988, 29: 11–16.
7. **Mulrooney SB, Hausinger RP:** Sequence of the *Klebsiella aerogenes* urease genes and evidence for accessory proteins facilitating nickel incorporation. Journal of Bacteriology 1990, 172: 5837–5843.
8. **Simor AE, Cooter NB, Low DE:** Comparison of four strains and a urease test for rapid detection of *Campylobacter pylori* in gastric biopsies. European Journal of Clinical Microbiology and Infectious Diseases 1990, 9: 350–352.
9. **Morotomi M, Hoshina S, Green P, Neu HC, LoGerfo P, Watanabe I, Mutai M, Weinstein IB:** Oligonucleotide probe for detection and identification of *Campylobacter pylori*. Journal of Clinical Microbiology 1989, 27: 2652–2655.
10. **Vandenberg FM, Zijlmans H, Langenberg W, Rauws E, Schipper M:** Detection of *Campylobacter pylori* in stomach tissue by DNA in situ hybridization. Journal of Clinical Pathology 1989, 42: 995–1000.
11. **Wetherall BL, McDonald PJ, Johnson AM:** Detection of *Campylobacter pylori* DNA by hybridization with non-radioactive probes in comparison with a ^{32}p-labelled probe. Journal of Medical Microbiology 1988, 26: 257–263.
12. **Valentine JL, Arthur RR, Mobley HLT, Dick JD:** Detection of *Helicobacter pylori* by using the polymerase chain reaction. Journal of Clinical Microbiology 1991, 29: 689–695.
13. **Clayton CL, Cleanthous H, Coates PJ, Morgan DD, Tabaqchali S:** Sensitive detection of *Helicobacter pylori* by using polymerase chain reaction. Journal of Clinical Microbiology 1992, 30: 192–200.
14. **Hammar M, Tyszkiewicz T, Wadstrom T, O'Toole PW:** Rapid detection of *Helicobacter pylori* in gastric biopsy material by polymerase chain reaction. Journal of Clinical Microbiology 1992, 30: 54–58.
15. **Clayton CL, Pallen MJ, Kleanthous H, Wren BW, Tabaqchali S:** Nucleotide sequence of two genes from *Helicobacter pylori* encoding for urease subunits. Nucleic Acids Research 1990, 18: 362.
16. **Wang JT, Wang TH, Sheu JC, Shih LN, Lin JT, Chen DS:** Detection of hepatitis B virus DNA by polymerase chain reaction in plasma of volunteer blood donors negative for hepatitis B surface antigen. Journal of Infectious Diseases 1991, 163: 397–399.
17. **Wang JT, Wang TH, Sheu JC, Lin SM, Lin JT, Chen DS:** Effect of anticoagulants and storage of blood samples on efficacy of the polymerase chain reaction assay for hepatitis C virus. Journal of Clinical Microbiology 1992, 30: 750–753.
18. **Kwok S, Higuchi R:** Avoiding false positives with PCR. Nature 1989, 339: 237–238.
19. **Graham DY, Lew GM, Klein PD, Evans DG, Evans DJ, Saeed ZA, Malaty HM:** Effect of treatment of *Helicobacter pylori* infection on the long-term recurrence of gastric or duodenal ulcer. A randomized, controlled study. Annals of Internal Medicine 1992, 116: 705–708.
20. **Rauws EAJ, Royen EAV, Langenberg W, Woensel JV, Vrij AA, Tytgat GN:** C-14-urea breath test in *Campylobacter pylori* gastritis. Gut 1989, 30: 798–803.

21. **Schnell GA, Schubert TT:** Usefulness of culture, histology and urease testing in the detection of *Campylobacter pylori* in gastric biopsies. American Journal of Gastroenterology 1989, 84: 133–137.
22. **Borromeo M, Lambert JR, Pinkard KJ:** Evaluation of "CLO-Test" to detect *Campylobacter pyloridis* in gastric mucosa. Journal of Clinical Pathology 1987, 40: 462–463.
23. **Newell DG, Rathbone BJ:** The serodiagnosis of *Helicobacter pylori* infection. Serodiagnosis and Immunotherapy of Infectious Diseases 1989, 3: 1–5.
24. **Goodwin CS, Armstrong JA:** Microbiological aspects of *Helicobacter pylori (Campylobacter pylori).* European Journal of Clinical Microbiology and Infectious Diseases 1990, 9: 1–13.