USE OF DIFFERENT PCR PRIMERS AND GASTRIC BIOPSY TISSUE FROM CLO TEST FOR THE DETECTION OF *HELICOBACTER PYLORI*

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Abstract. Four different DNA loci were assessed for the detection of *H. pylori* by PCR on gastric biopsy specimens. PCR, with a primer specific 860 bp DNA fragment, was the most sensitive, with a detection limit of 0.02 pg *H. pylori* DNA, corresponding to approximately 10 organisms. Nested-PCR of the 860-bp DNA fragment was 10-fold more sensitive than single-step PCR. The sensitivity and specificity of the four PCR methods, in comparison to the results obtained from histology and the urease test, are as follows: 80.7% and 76% for the *hpaA* gene; 100% and 76% for the 16S rRNA gene; 84.6% and 80.0% for the 860-bp DNA fragment; 61.5% and 84.0% for the *ureC (glmM)* gene, respectively. The sensitivity of nested-PCR for the 860-bp DNA fragment was 100%. This nested-PCR gave positive results for eight specimens which were negative by conventional methods. PCR can be performed on gastric biopsy specimens obtained from the CLO test.

INTRODUCTION

Helicobacter pylori (H. pylori) is a bacterium found in the gastric mucosa of patients with peptic ulcer disease (Marshall, 1984). The organism plays an important role in the pathogenesis of gastritis, peptic ulcer disease and gastric carcinoma (Goodwin *et al*, 1997). In developing countries, approximately 50-90% of the population is infected with *H. pylori* (Perez-Perez *et al*, 1990; The EUROGAST Study Group, 1995; Peerakome *et al*, 1996). It has been shown that the elimination of *H. pylori* may reduce the risk of peptic ulcer disease and gastric carcinoma and is the most cost effective long term treatment for and prevention of these problems (Walsh and Perterson, 1995). A simple, accurate, sensitive, and less time consuming method of detecting *H. pylori* as a means of monitoring the efficacy of therapy is required. Direct methods used to diagnose *H. pylori* infection require the collection of gastric samples by biopsy during endoscopy, followed by cultivation, histologic examination and urease detection. Serological tests and the urea breath tests are indirect techniques for the detection of *H. pylori* infection. Some techniques, such as culture, histology, and the urea breath test, have been used as gold standards (Goodwin *et al*, 1997). However, the success of these techniques depends on local expertise and access to facilities. The polymerase chain reaction (PCR) is a powerful research tool, that has become part of the routine clinical laboratory in the fields of microbiology, virology, and genetic diagnosis. PCR is very sensitive and can detect the presence of an organism at very low levels. The sensitivity of the PCR varies with the target, type of PCR, and detection system used. Several PCR assays have been developed to detect *H. pylori* DNA in clinical specimens and have proved to be highly sensitive compared to the conventional assays (Ho *et al*, 1991; Valentine *et al*, 1991; Claton *et al*, 1992).

In our study, PCRs that target four differ-

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ent DNA loci were evaluated for the detection of *H. pylori* on gastric biopsy specimens. The sensitivities and specificities of the PCR methods were compared to those of other methods. In addition, we also looked at the potential use of the same pieces of tissue used for the urease test for assuring the presence of *H. pylori* by PCR.

MATERIALS AND METHODS

Tissue specimens

The specimens used for the study were gastric biopsy samples from patients whom had undergone endoscopy at Uttaradit Hospital, Uttaradit, Thailand for the diagnosis of abdominal pain. The biopsy specimens were the same pieces of tissues used for CLO test (Marshall *et al*, 1987). Twenty-seven CLO test-positive and 25 CLO test-negative gastric biopsy specimens were used after the test was performed. *H. pylori* DNA was extracted using the QIAamp DNA Mini Kit (QIAGEN, Germany). The DNA extraction samples were stored at -20ºC until PCR amplification.

Other specimens

Other DNA from gastric biopsy specimens was obtained from patients whom had undergone endoscopic examinations at Maharaj Nakhon Chiang Mai Hospital, for which the results of histology and urease tests were available (Linpisarn *et al*, 2003).

PCR primers and amplification

The target genes, sizes of PCR products, primer sequences, and PCR amplification conditions are summarized in Table 1. Each sample was examined with four different PCR primer sets. The PCRs were performed as described elsewhere (Linpisarn *et al*, 2003) Briefly, 10 μl PCR mixture contained 1 μl DNA extraction product, PCR buffer [20mM Tris-HCl (pH 8.4), 50 mM KCl, 3.0 mM $MgCl_2$, 0.01% BSA and 0.05% Tween], 0.2 mM (each) deoxynucleotide triphosphates, 0.25 U of *Tag* DNA polymerase, 0.25 μM primer Hpa1/Hpa2 for the *hpaA* gene, primer Hp1/Hp2 for 16S rRNA, 0.125 μM primer UF/UR for *ureC (glmM)* and 0.5 μM primer EHC-L/EHC-U for the 860-bp DNA fragment. The PCR products were electrophoresed on 2% agarose gels. Samples were scored as positive when a band of the expected size, as summarized in Table1, could be detected on the gel. *H. pylori* genomic DNA from culture provided by Dr Carl J Mason, The Armed Forces Research Institute of Medical Sciences (AFRIMS) Bangkok, Thailand, was used as a positive control. Negative reagent controls containing distilled water instead of the DNA sample were included in each batch of the amplifications. Specific amplicons were sequenced in both directions using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit. The sequence comparison was carried out using the DNAsis program and the Genbank databases.

Nested-PCR was performed based on the 860-bp DNA fragment sequence. The nested primer for ET-5U was: 5′- GCC AAA TCA TAA GTC CGC AGA A-3′ and for ET-5L was: 5′- TGA GAC TTT CCT AGA AGC GGT GTT-3′, which yielded a 230 bp PCR product (Song *et al*, 1999). The PCR for the first round was as described above except only 20 cycles were used. One percent of the first reaction product served as the template for the second PCR in the 35 amplification cycles.

Sensitivity of the PCR methods

DNA from *H.pylori* culture was serially diluted at 1:10 from 2 ng to 0.02 fg. Each dilution was examined by all four PCRs.

Statistical analysis of data

Sensitivity, specificity, positive and negative predictive values (PPV and NPV, respectively) were calculated according to standard methods.

RESULTS

PCR detection of *H. pylori* on the gastric biopsy specimens with primers specific for *hpaA*, 16S rRNA, the 860-bp DNA fragment and the *ureC (glmM)* genes gave products of the expected size (Fig 1). The DNA sequences of the PCR products were found to be 95-99% identical to the published sequence for *H. pylori* [GenBank accession number X61574 for the *hpaA* gene; number Z25741 for the16S rRNA;

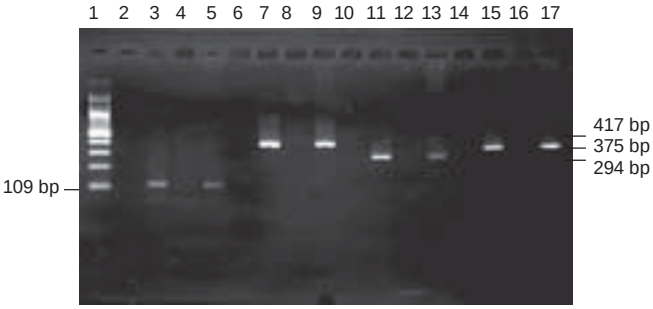

Fig 1–Detection of *H. pylori* on gastric biopsy samples by a single-step PCR with primers Hp1/Hp2 for 16S rRNA (lanes 2-5); EHC-L/EHC-U for 860-bp DNA fragment (lanes 6-9); UF/UR for *ureC (glmM)* (lanes 10-13); Hpa-1/Hpa-2 for *hpaA* gene (lanes 14-17); lane 1: 100 bp DNA ladder; lanes 2,6,10 and 14: negative controls; lanes 3,7,11 and 15: *H. pylori* genomic DNA; lanes 4,8,12 and 16: negative samples; and lanes 5,9,13,17: positive samples.

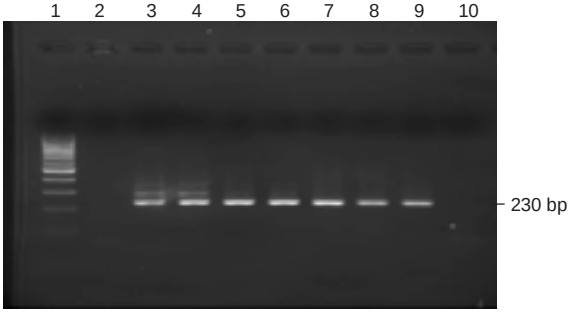

Fig 2–Sensitivity of the nested-PCR with primers EHC-L/EHC-U and ET-5U/ET-5L for 860-bp DNA fragment. Lane 1: 100 bp DNA ladder; lane 2: negative control; lanes 3-10: 10-fold serial dilutions of *H. pylori* DNA extracted from cultured cells (2.0 ng-0.2 fg).

number NC_000915 for the 860-bp DNA fragment; and number AF405553 for *ureC (glmM)*].

The sensitivity for each set of primers was determined based on the *H. pylori* DNA obtained from the culture. The primer specific for the 860-bp DNA fragment gave the highest sensitivity, detecting as little as 0.02 pg of *H. pylori* DNA, corresponded to approximately 10 organisms. The nested-PCR for the 860-bp DNA fragment was 10-fold more sensitive than the single-step PCR (Fig 2).

The results of the PCR methods with the four different primer sets to detect *H. pylori* on gastric biopsy specimens were compared to the histology and urease tests (Table 2). The primers Hp1/Hp2 specific for the 16S rRNA gene were 100% sensitive and 76% specific. The *ureC (glmM)* gene PCR had a high specificity (84%) but a low sensitivity (61.5%). For the primer EHC-U/EHC-L specific for the 860-bp DNA fragment, the sensitivity and specificity were 84.6% and 80.0%, respectively. These primer sets detected *H. pylori* in five specimens which gave negative results on histology and the urease test (sample numbers 3, 11, 41, 42, and 52). Nested-PCR with the primer ET-5U/ET-5L gave a 230 bp product; the sensitivity and specificity were 100% and 68%, respectively.

The results of *H. pylori* detection by the three PCR methods were compared with those of the CLO test, which was performed on the same gastric biopsy specimens. The sensitivities and specificities of the PCRs with the different primer sets ranged from 80.0 to 92.0%

Table 1
Target genes, product size, sequences of the four different primer sets and PCR conditions.

Target gene	Product size (bp)	Primer sequences	PCR conditions	Reference
hpaA	375	Hpa-1, GA ATT ACC ATC CAG CTA GCG Hpa-2, GT AAC CTT GAC AAA ACC GGC	94ºC, 30s; 56ºC, 1min; 72ºC, 1 min (35 cycle)	Hulten *et al*, 1996
16S rRNA	110	Hp1, CTG GAG AGA CTA AGC CCT CC Hp2, ATT ACT GAC GCT GAT TGT GC	94ºC, 30s; 55ºC, 1min; 72ºC, 1 min (40 cycle)	Ho *et al*, 1991
860-bp DNA fragment	417	EHC-L, AAG AAG TCA AAA ACG CCC CAA AAC EHC-U, CCC TCA CGC CAT CAG TCC CAA AAA	94ºC, 30s; 55ºC, 1min; 72ºC, 1 min (40 cycle)	Song *et al*, 1999
ureC (glmM)	294	UF, AAG CTT TTA GGG GTG TTA GGG GTT UR, AAG CTT ACT TTC TAA CAC TAA CGC	94ºC, 30s; 57ºC, 1min; 72ºC, 1 min (35 cycle)	Lu *et al*, 1999

Table 2
H. pylori detection on gastric biopsy specimens by different PCR methods, histology and the urease test.

PCR method Target gene (primer)	Histology and urease test results	
	H. pylori positive (Patient no.)	*H. pylori* negative (Patient no.)
hpaA (Hpa-1/Hpa-2)		
Positive	21	6 (3,42,44,47,48,52)
Negative	5 (5,16,29,38,59)	19
16S rRNA (Hp1/Hp2)		
Positive	26	6 (3,11,42,49,52,65)
Negative	0	19
860-bp DNA fragment (EHC-L/EHC-U)		
Positive	22	5 (3,11,41,42,52)
Negative	4 (57,59,61,69)	20
860-bp DNA fragment (ET-5U/ET-5L) Nested-PCR		
Positive	26	8 (3,11,41,42,47,52,62,65)
Negative	0	17
ureC (glmM) (UF1/UF2)		
Positive	16	4
Negative	10	21

Table 3
Sensitivity, specificity, positive and negative predictive values of the three PCR methods for *H. pylori* detection using gastric sample from the CLO test compared to the CLO test.

Target gene (primer)	Sensitivity (%)	Specificity (%)	Predictive value (%)	
			Positive	Negative
hpaA (Hpa-1/Hpa-2)	80	81.8	83.3	78.3
16S rRNA (Hp1/Hp2)	92	90.9	92	90.9
860-bp DNA fragment (EHC-L/EHC-U)	88	81.8	84.6	85.7

and 81.8 to 90.9%, respectively (Table 3).

DISCUSSION

This study assessed four PCR methods for the detection of *H. pylori* on gastric biopsy specimens. The chosen targets for these PCR methods included the 16S rRNA, which has been the most widely used and has been proven to have a high sensitivity (Ho *et al*, 1991; Mapstone *et al*, 1992; van Zwet *et al*, 1993; Weiss *et al*, 1994). The *hpaA* PCR can detect as little as two bacterial cells and has been used to confirm the presence of *H. pylori* in drinking water (Hulten *et al*, 1996). The 860-bp DNA fragment PCR has been shown to be highly sensitive and specific for the detection of dental plague and saliva (Song *et al*, 1999, 2000). In addition, Lu

et al (1999) compared the sensitivities and the specificities of five PCR methods for the detection of *H. pylori* on gastric biopsy specimens and demonstrated that the *ureC (glmM)* gene PCR was the most appropriate method.

The results of our study show that the DNA sequences of different PCR amplifications demonstrated some strain diversity compared with the Western isolates. However, the PCR primers in our study were taken from well conserved regions (data not shown). The 860-bp DNA fragment PCR resulted in the highest sensitivity and detected 0.02 pg *H. pylori* DNA. The sensitivity and specificity of the 860-bp DNA fragment PCR compared to the results of histology and urease test; demonstrating a high sensitivity (84.6%) and specificity (80.0%). The nested-PCR for the 860-bp DNA fragment was 10-fold more sensitive than a single-step PCR, which corresponded to one bacterial genome. These results are comparable to those obtained from another study (Song *et al*, 1999). The sensitivity and specificity of the nested-PCR for the detection of *H. pylori* in gastric biopsy specimens was 100% and 68%, respectively. The low specificity may be explained by the positive results in 8 samples which were negative by conventional methods. The nested-PCR detected 4 samples which gave negative results for a single-step PCR (sample nos. 57, 59, 61, and 69).

It has been recommended that for developing countries and for daily practice, either the CLO test or Giemsa stain of biopsy specimens is sufficient to identify *H. pylori* (Suwanagool *et al*, 1993). Cutler *et al* (1995) suggested that when the CLO test results are positive, there is no diagnostic benefit to performing additional tests. However, when the CLO test is negative and there is a suspicion of *H. pylori*, histological staining methods should performed. In our study, *H. pylori* DNA was detected by PCR on gastric biopsy material obtained from the CLO test. The PCR, which proved to be specific and sensitive for the detection of *H. pylori* DNA can supplement histology. The advantage of using the gastric biopsy specimens from the CLO test is to reduce the number of biopsies performed during endoscopy. *H. pylori* genotyping, such as the *cagA, vacA* and *iceA* subtypes, can be determined routinely from a positive CLO test.

In conclusion, our results demonstrate nested-PCR, with primers specific for the 860-bp DNA fragment, is the most appropriate PCR method for the detection of *H. pylori* on gastric biopsy specimens. The PCR method can detect *H. pylori* DNA in gastric biopsy material obtained for the CLO test.

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