Detection of *Helicobacter pylori* in Gastric Biopsy Samples by Polymerase Chain Reaction with a Simple DNA Extraction Method

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ABSTRACT

We have established a PCR assay for the detection of Helicobacter pylori in gastric biopsy specimens with primers specific to adhesin subunit gene. The minimum amount of H. pylori DNA that could be detected was 0.4 pg. Seventy-two antral biopsy specimens were taken from patients with gastritis and/or duodenal ulcer. H. pylori was found in 58.3%, 44.4% and 54.2% of patients according to the results of histology, urease test and PCR assay respectively. The sensitivity and specificity of PCR assay, compared to histology technique were 66.7% and 63.3% respectively. Samples diagnosed to be positive for H. pylori by both histology and urease test were only 85% PCR positive. Specimens that were negative in both tests were also PCR negative (72%).

DNA was extracted by two methods: the Chelex chelating resin method and the Qiagen DNA Mini Kit. The sensitivity and specificity of the PCR assay, using the Chelex-extracted DNA as templates were 85.7% and 85% respectively as compared to those extracted with the kit.

Key words: *Helicobacter pylori*, Polymerase chain reaction, Gastric biopsy, Adhesin subunit gene, Chelating resin

INTRODUCTION

Helicobacter pylori (H. pylori) has been recognized to play an important role in the pathogenesis of gastritis and peptic ulcer disease (Goodwin et al., 1997). The eradication of *H. pylori* has become an accepted therapy in order to prevent the relapse of the disease (NIH consensus statement; 1994).

There are several methods described for diagnosis of *H. pylori* infection. Endoscopy is used to obtain biopsy material for histological sections that are stained with Giemsa. Another biopsy-based technique is to culture the bacteria, but this is demanding as the organism is fastidious and its growth occurs at best after 3 days. *H. pylori* produces an enzyme urease

which can be tested in biopsy material. This urease test is simple and specific but has a low sensitivity. Urease can also be tested non-invasively with the urea breath test that uses radioisotopes and a specific instrument that makes this test too expensive for use in developing countries. Detection of antibodies against *H. pylori* in serological tests provides no real answer to the time and the current status of infection, especially after an eradication treatment (Moss et al., 1998). On the other hand, the polymerase chain reaction (PCR) proved useful in the detection of a number of pathogens. PCR tests are usually highly sensitive, specific and fast. So, it is not surprising that there are several PCR-based approaches described for various sites in the genome of *H. pylori* (Valentine et al., 1991; Claton et al., 1992; Weiss et al., 1994).

The important step for the PCR technique is the extraction of DNA from gastric biopsy specimens. The classical method is a phenol-chloroform extraction. However, it is quite time-consuming and creates chemical wastes hazardous to health. In this study, we established a PCR assay for the detection of *H. pylori* in gastric biopsy specimens with primers specific to the adhesin subunit gene. We also compared two methods of extracting *H. pylori* DNA from gastric biopsy: a silica gel membrane method used in a commercially-available kit (QIAamp® DNA Mini Kit) and chelating resin, Chelex-100.

MATERIALS AND METHODS

Biopsy materials

Gastric biopsy materials were obtained from 72 patients with gastritis and/or duodenal ulcer who underwent endoscopic examinations at the Gastroenterology Unit, Maharaj Nakorn Chiang Mai Hospital, Chiang Mai, Thailand. Four antral biopsy specimens were collected from each patient. One specimen was tested for the presence of urease enzyme and another was fixed in formalin for histological examination. The remaining two specimens were kept at -20 ˚C for the detection of *H. pylori* by PCR.

The study was approved by the Human Experimentation Committee, Research Institute for Health Sciences, Chiang Mai University, Thailand.

DNA extraction

H. pylori genomic DNA from culture was provided by Dr. Carl J. Mason, The Armed Forces Research Institute of Medical Sciences (AFRIMS), Bangkok, Thailand. DNA from one biopsy specimen was extracted solely by using the QIAamp® DNA Mini Kit (QIAGEN, Germany). In parallel, DNA was extracted using the Chelex-100 chelating resin as modified from elsewhere (Hoff-Olsen et al., 1999). The Chelex-100 extracting procedure was as follow: one piece of gastric biopsy specimen was washed with sterile water 3 times and then covered with chelex beads from an aqueous suspension. The sample was incubated overnight at 56 ˚C and boiled for 5 minutes.

Primers and PCR conditions

PCR was carried out with primers specific to the adhesin subunit gene. The two primers were selected from those reported by Hulten et al., (1996), Hpa 1 (5'- GA ATT ACC ATC CAG CTA GCG - 3') and Hpa2 (5'- GT AAT CTT GAC AAA ACC GGC - 3') which yielded the expected product of 375 nucleotide pairs in length.

Two microlitres of DNA extraction was added to 8 microlitres of PCR mixture (20mM Tris pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% BSA, 0.05% Tween, 0.2 mM of each dNTP, 0.25 U Taq polymerase, 0.25 μM of each primer and 10% glycerol). Thirty-five cycles were employed and each cycle consisted of 30 seconds denaturation at 95 ˚C, 1 minute annealing at 56 ˚C and 1 minute extension at 72 ˚C. After 35 cycles, the reaction mixture was further extended for 7 minutes at 72 ˚C.

The PCR products were analyzed by agarose gel electrophoresis. Eight microlitres of PCR product were run on a 2% agarose gels, containing ethidium bromide and photographed under UV light. A 375-bp band was considered a positive PCR result. The specific amplicon was confirmed by digestion with restriction enzyme Hinf I and also DNA sequencing. A negative control (sterile water) and a positive control (*H. pylori* genomic DNA) were included in each run of amplifications.

The sensitivity of the established PCR was evaluated by 10-fold serial dilutions of *H. pylori* genomic DNA (ranging from 0.5 fg/μl to 2 ng/μl).

Diagnosis of *H. pylori by* conventional methods

The biopsy specimens from the antrum of each patient were examined by rapid urease test, using the in-house-made kit (Phornphutkul et al., 1998). The development of a red colour within 2 hours after placing biopsy specimen into the media indicated a positive result. Histological examination was also performed, using standard stains (hematoxylin-eosin and Giemsa) and assessed by a pathologist.

RESULTS

Amplification of *H. pylori* genomic DNA gave the expected 375 bp fragment. Digestion of PCR products with the restriction enzyme Hinf I generated expected specific fragments of 259 bp and 116 bp (Figure1). PCR products were also confirmed by sequencing and the obtained sequences were compared to those obtained from Genbank sequence (accession X 61574). The assay could detect as little as 0.4 pg of *H. pylori* DNA which corresponded to 200 genome copies of *H. pylori.*

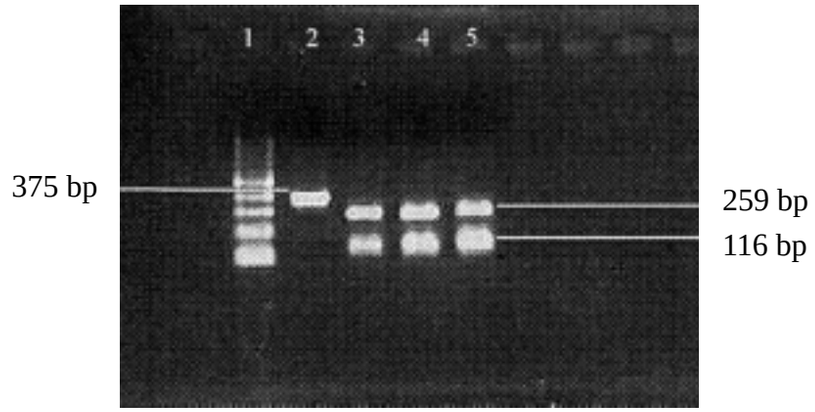

Figure 1. Confirmation of the PCR products by digesting with restriction enzyme Hinf I. Lane1: 100 bp DNA ladder; Lane2:PCR product from H. pylori genomic DNA; Lane3-5: PCR product from digestion with different concentrations of Hinf I, 20,30 and 40 unit.

Individual results obtained from different methods of detection of *H. pylori* are shown in Table1. *H. pylori* from seventy-two antral biopsy specimens was detectable by histology, urease test and PCR assay in 58.3%. 44.4% and 54.2% of the cases respectively (Table 1). The PCR assay could detect this organism in seven specimens which were negative in both histology and urease test. The sensitivity and specificity of the established PCR, compared to histology technique were 66.7% and 63.3% respectively (Table 2). However, samples diagnosed to be positive for *H. pylori* by both histology and urease test. PCR could detect correctly 85% of the positive samples whereas specimens that had negative results in both tests, PCR gave negative results of 72%.

Table 1. *H. pylori* in gastric biopsy specimens examined by histology method, urease test and PCR assay.

*Specimen number	Histology	Urease test	PCR
1 to 18 (18)	-	-	-
19 to 41 (23)	+	+	+
42 to 45 (4)	+	+	-
46 to 50 (5)	+	-	+
51 to 60 (10)	+	-	-
61 to 64 (4)	-	+	+
65 (1)	-	+	-
66 to 72 (7)	-	-	+
Positive results (%)	42/72 (58.3%)	32/72 (44.4%)	39/72 (54.2%)

*Arbitrary number; all tests were performed on each gastric biopsy.

Table 2. Sensitivity, specificity, positive predictive value(PPV), and negative predictive value (NPV) of each assay with histologic technique as a gold standard.

	PCR	Urease test
Sensitivity (%)	66.7	64.3
Specificity (%)	63.3	83.3
PPV (%)	71.8	84.4
NPV (%)	57.6	62.5

The PCR products from the *H. pylori* DNA which were extracted by using Chelex-100 chelating resin were in good agreement with those obtained by the QIAamp®DNA Mini Kit (Figure 2). The sensitivity and specificity of the detection of H. pylori by PCR using DNA template from Chelex-100 extraction were 85.7% and 85% respectively when compared to the kit (Table 3).

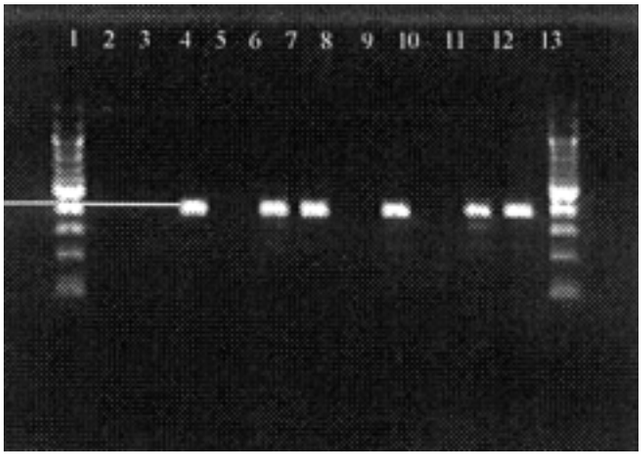

Figure 2. Detection of *H. pylori* in gastric biopsy specimens by PCR: Comparison of DNA extraction methods; QIAamp®, PCR and Chelex-100. Lane 1,13: 100 bp DNA ladder; Lane 2: negative control; Lane 3-7: DNA template extracted by QIAamp®; Lane 8-12: DNA template extracted by Chelex-100.

Table 3. Comparison of detection of H. pylori by PCR using DNA template obtained from Chelex-100 and QIAamp® DNA Mini Kit extracting method.

Method and results		QIAamp® DNA Minikit		
		Positive	Negative	Total
Chelex-100	Positive	24	4	28
	Negative	3	17	20
Total		27	21	48

DISCUSSION AND CONCLUSION

It is well accepted that PCR is a highly-sensitive technique for the detection of several pathogens. Therefore, the most important factor is to minimize the potential contamination. In our study, extreme care was taken to prevent *H. pylori* DNA samples from contamination. We performed sample collection, DNA extraction, pre-PCR preparation, PCR amplification and post-amplification analysis in separate rooms. The pre-PCR preparation room was equipped with a laminar flow cabinet with an UV lamp. This work area had its own pipettes, rack, tube and other disposal items. Furthermore, we used filtered pipette tips in order to prevent aerosal contamination of pipettes. False positive results may come from amplicon carry-over. Therefore, a negative control using sterile water instead of DNA template was included in each run. We feel confident that the results obtained from our study are reliable.

The established PCR could detect *H. pylori* DNA concentration of 0.4 pg or approximately equivalent to 200 cells of bacteria. The sensitivity which we obtained was lower than that reported elsewhere (Taylor et al., 1992; Lage et al., 1995; Hulten et al., 1996). Several factors may contribute to a low sensitivity such as the different detection methods of PCR product. Moreover, the PCR primers used in this study were designed for the detection of a *H. pylori* strain isolated from American populations (Evans et al., 1993). Since it is

known that there is some sequence variation between different *H. pylori* strains, there was the possibility that the primers did not properly bind to the particular *H. pylori* strain encountered in Chiang Mai. Hulten et al., (1996) designed primers specific to the adhesin subunit gene of *H. pylori* and detected the PCR product by Southern blot hybridization, thereby they could detect as little as a single cell. Song et al., (1999) demonstrated a difference in specificity and sensitivity of the PCR assay for *H. pylori* with different primer sets. In addition, Kawamata et al., (1996) reported that the primers targeting the *H. pylori* urease gene were designed, based on the sequence conservation analysis of sixteen *H. pylori* strains isolated from Japanese patients and proved to have excellent sensitivity and specificity. The purpose of our study is to establish a PCR assay for the detection of *H. pylori* for diagnosis in routine laboratory. Therefore, the procedure should be simple and requires a minimum number of steps.

The results of this study demonstrate that a PCR assay could detect *H. pylori* in gastric biopsy specimen obtained from patients with gastritis and/or duodenal ulcer. However, the sensitivity and specificity of the PCR assay when compared to histology as a gold standard was only 66.7% and 63.3% respectively which is much lower than in the other studies (Valentine et al., 1991; Lage et al., 1995; Thijs et al., 1996). There were ten specimens which gave positive results by histology, but the results from PCR and urease test were negative. We have observed that all of these specimens showed either scanty or grade +1 of histology results. Furthermore, four specimens which were positive by the histology and urease test showed scanty or mild positive PCR results. It is possible that patients with low amount of bacteria in the tissue may contribute to the inconsistency of *H. pylori* in each piece of gastric biopsy specimens. Nevertheless, the PCR assay could detect *H. pylori* from an additional seven biopsy specimens which were negative on both histology and urease test. This means that the PCR assay can be more sensitive than the other methods under certain conditions. The sensitivity and specificity of the assay could be improved by designing primers specific to the local Thai strain of *H. pylori* and developing a nested-PCR. The Chelex-100 chelating resin produced a suitable DNA template for *H. pylori* detection by PCR and the extraction procedure is simple, rapid and the cost of reagents is relatively low.

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