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# Utilization of Nested-PCR for Confirmation of *H. pylori* Detection by CLO® Test

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## ABSTRACT

***Background:*** In Thailand, direct demonstration of *H. pylori* infection in gastric mucosa specimens, such as mucosal culture and mucosal histological examination, is usually recommended. However, in resource-limited settings where adequate culture facilities and expertise are lacking, false negative results may often be a consequence. CLO® test is widely used in clinical practice, but may also give false negative results. The aim of this study was to utilize the nested-PCR for confirmation of *H. pylori* detection by CLO® test.

***Materials and Methods:*** A total of 122 patients who underwent esophagogastroduodenoscopy at Uttaradit Hospital between October 2003-2005 were enrolled and completed the study. Gastric biopsy specimens were taken and sent for culture, histologic examination, rapid urease test and nested-PCR assay.

***Results:*** There were 73 males and 49 females (mean age 57 ± 14.8, range 18 to 85 yrs.). Endoscopic findings revealed gastritis in 72 (59%), gastric ulcers in 35 (28.7%), duodenal ulcers in 12 (9.8%) and combined ulcers in 3 patients (2.4%). *H. pylori* were detected in 37, 43, 93, and 96 samples by culture, histology, urease test and PCR technique, respectively. PCR technique yielded a sensitivity of 97.8%, which is higher than other methods. The sensitivities of culture, histology and rapid urease test were 39.8%, 45.2% and 96.8%, respectively. However, PCR technique was associated with a specificity of 82.8%, lower than 100% by culture and 89.7% by rapid urease test, while the specificity of histology was 69.5%. Both CLO® test and PCR assay gave highly accurate results.

***Conclusions:*** Results from this study demonstrated high sensitivity, specificity and accuracy of PCR technique for detecting *H. pylori* infection. This technique offers another option for a good diagnostic tool in such a limited resource setting as ours.

**Key words :** Nested-PCR, *H. pylori* infection

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## INTRODUCTION

It is now generally accepted that *Helicobacter pylori (H. pylori)* infection can be cured, and eradication of the organism can dramatically reduce the relapse rate and complications of gastritis and peptic ulcer disease, as well as favorably altering the natural course of gastric MALT lymphoma, gastric adenocarcinoma and immunologic thrombocytopenia[1]. Direct methods of detecting *H. pylori* from gastric tissue have proven to be sensitive and specific[2]. These methods are appropriate in patients with dyspepsia or upper gastrointestinal tract (UGI) bleeding, or other alarming conditions requiring are endoscopic examination. Rapid urease test, histological examination by Hematoxylin & Eosin (H&E) or Giemsa stain, and techniques for culturing bacteria from gastric tissue are generally regarded as standard methods for detecting *H. pylori* bacterium[3]. However, in limited resources settings, lack of expertise and inadequate laboratory and clinical facilities may lead to significant false negative results. PCR-based technique is of interest in the detection of small amounts of the bacteria. In this study, the sensitivity and specificity of PCR-based technique was compared to conventional methods.

## MATERIALS AND METHODS

### Patients

A total of 122 patients who underwent esophagogastroduodenoscopy (EGD) at Uttaradit Hospital in northern Thailand between October 2003 and October 2005 were recruited into the study. Exclusion criteria were previous gastric surgery, prior use of proton pump inhibitors or antibiotics within the preceding one month, and patients with coagulopathy. EGD was performed by one endoscopist, using either Olympus GIF (Olympus optical Co. Ltd., Tokyo, Japan) or Pentax EG2970K (Pentax Co. Ltd., Tokyo, Japan) videoendoscope. Four antral biopsy specimens were taken within 3 cm. of the pyloric sphincter, one for rapid urease test, one for histological examination, one for culture and one for PCR assay. A written informed consent was obtained from every patient before enrollment.

### Diagnostic assays

1) Rapid urease test: Biopsy specimens were put into CLO® test gel (Ballard Medical Products, Draper, Utah, USA). The color change was monitored up to 24 hours at room temperature; a positive result was identified if the color changes from yellow to pink.

2) Histology: Biopsy specimens were fixed with 10% formalin solution and stained with H&E and Giemsa. Inflammation score was histologically classified into mild, moderate and severe. The presence of intestinal metaplasia and curved organisms were also observed.

3) Culture: Biopsy specimens were kept in trypticase soy broth under cold condition and immediately transported to the hospital microbiology laboratory. The gastric specimens were smeared onto plate of Skirrow media (CAMP Selectavial (Skirrow), Mast Diagnostics, England) and incubated under microaerobic condition (Gas generating kit anaerobic and $CO_2$ system, Oxoid, England) for 4 days. Colonies were recorded and subjected to gram-stain and biochemical tests, including urease, catalase and oxidase tests. *H. pylori* positivity was identified if colonies were small, round and shiny along side the presence of gram-negative curved bacilli, and all three tests were positive[4].

4) PCR technique: Samples were frozen biopsy specimens or those removed from the gel of the CLO® test after color change was recorded. The sample was processed for nested-PCR analysis following the previously described method[5]. Briefly, gastric biopsy specimens were transferred to microcentrifuge tubes and washed twice with distilled water. *H. pylori* DNA was extracted using QIAamp DNA MiniKit (QIAGEN, Germany). The DNA extraction samples were stored at -20 ˚C until PCR amplification. PCR target was 860 bp fragment and the two primers pair used were EHC-L; AAG AAG TCA AAA ACG CCC CAA AAC and EHC-U; CCC TCA CGC CAT CAG TCC CAA AAA. PCR was carried out in 10 µl volume containing 1 µl DNA extraction product, PCR buffer (20 mM 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 Taq DNA Polymerase and 0.5 µM primer. Twenty cycles of amplification were performed in DNA thermocycler (Gene Amp 9700 system, AB Applied Biosystem, USA). Each cycle consisted of a 30 sec denaturation step at 94 ˚C, a 1 min annealing step at 55 ˚C and 1 min extension step at 72 ˚C. The final cycle was a 7 min extension step at 72 ˚C to ensure full extension of the product. After PCR, one percent of the first reaction product was transferred to the second round reaction mixture and reamplified with the nested pair of primers of the following sequences, ET-5U;GCC AAA TCA

TAA GTC CGC AGA A and ET-5L; TGA GAC TTT CCT AGA AGC GGT GTT. The PCR condition was the same as described above except that 35 cycles was used for the second PCR reaction. The PCR products were analyzed by agarose gel electrophoresis and the expected nested-PCR product was 230 bp. A negative control (sterile water) and a positive control (*H. pylori* genomic DNA) were included in each run of amplifications.

## Gold standard

The patients were considered as *H. pylori* positive if culture gave positive results, or positive results were obtained in any two of the following assays: histology, urease test or PCR. Negative *H. pylori* patients were defined by a negative culture result and at least any two negative results from histology, urease test or PCR assays[6,7].

## Statistics

Descriptive analysis was used for demographic and clinical features as mean and percentage. Sensitivity, specificity, accuracy, positive and negative predictive values were determined for each diagnostic test.

## RESULTS

A total of 122 patients (73 males, 49 females) with age ranging from 18 to 85 years (mean 57 ± 14.8) were included in the study. The endoscopic findings showed gastritis in 72 (59%), gastric ulcers in 35 (28.7%), duodenal ulcers in 12 (9.8%) and combined ulcers in 3 (2.4%) patients (Table 1). After a short run, we decided to use the gastric specimen taken from the already read CLO® test gel for PCR technique (83/122). One-hundred-and-two patients were positive for *H. pylori* by at least one method, while 92 patients had 2 or more positive tests (Table 2). The positive tests for *H. pylori* as detected by culture method, histology, rapid urease test and PCR technique were 37, 43, 93 and 96, respectively (Table 3).

Eighty-nine biopsy samples were positive and 22 were negative by both the CLO® test and the nested-PCR assay. However, 11 biopsy samples gave contradictory results, whereby 4 samples demonstrated negative PCR but positive CLO® test, while 7 positive for PCR were negative by CLO® test (Table 4).

There were 93 (76.2%) patients who had *H. pylori* infections. The culture method gave the lowest sensitivity of 39.8%, but showed the highest specificity of 100%. Histology had low sensitivity of 45.2% but also high specificity of 96.5%. CLO® test and PCR technique had sensitivity of 96.8% and 97.8%, and specificity of 89.7% and 82.8%, respectively. Both CLO® test and PCR assay gave highly accurate results (95.1% vs 94.3%) (Table 5).

**Table 1** Characteristics of the patients

| | |
|---|---|
| Total | N = 122 |
| Gender M/F | 73/49 |
| Age (years) | 57 ± 14.8 (range 18-85) |
| Gastritis | 72 (59%) |
| Gastric ulcers | 35 (28.7%) |
| Duodenal ulcers | 12 (9.8%) |
| Combined ulcers | 3 (2.4%) |

**Table 2** Number of positive test for *H. pylori* by four diagnostic methods

| Number of Positive Tests | Number of patients (percent) |
|---|---|
| 0 | 20 (16.4%) |
| 1 | 10 (8.2%) |
| 2 | 38 (31.1%) |
| 3 | 33 (27.0%) |
| 4 | 21 (17.2%) |

Four diagnostic methods = Culture, Histology, Urease test and PCR

**Table 3** Results of each test compared to gold standard

| Tests | Gold standard | |
|---|---|---|
| | Positive | Negative |
| Culture | | |
| Positive | 37 | 0 |
| Negative | 56 | 29 |
| Histology | | |
| Positive | 42 | 1 |
| Negative | 51 | 28 |
| Urease test | | |
| Positive | 90 | 3 |
| Negative | 3 | 26 |
| PCR | | |
| Positive | 91 | 5 |
| Negative | 2 | 24 |

**Table 4** Results of bacterial culture, histology, urease test and nested-PCR for the detection of *H. pylori*

| Sample ID | Culture | Histology | Urease test | Nested-PCR |
|---|---|---|---|---|
| #22 | - | - | + | - |
| #55 | - | - | + | - |
| #56 | - | - | + | - |
| #116 | + | - | + | - |
| #24 | - | - | - | + |
| #33 | - | - | - | + |
| #34 | + | + | - | + |
| #40 | + | + | - | + |
| #41 | - | - | - | + |
| #49 | - | - | - | + |
| #53 | - | - | - | + |

+ = positive result, - = negative result

**Table 5** Sensitivity, specificity, accuracy, positive and negative predictive values (PPV, NPV) of each method

| | Culture | Histology | Rapid urease test | PCR |
|---|---|---|---|---|
| Sensitivity (%) | 39.8 | 45.2 | 96.8 | 97.8 |
| Specificity (%) | 100 | 96.5 | 89.7 | 82.8 |
| PPV (%) | 100 | 97.7 | 96.8 | 94.8 |
| NPV (%) | 34.1 | 35.4 | 89.7 | 92.3 |
| Accuracy (%) | 54.1 | 57.4 | 95.1 | 94.3 |

## DISCUSSION

In this study, direct methods for detection of *H. pylori* in gastric biopsy specimens were compared: culture of the bacteria, histological examination, rapid urease test and nested-PCR. All four methods were concurrently in agreement in only 16.4% of negative cases and 17.2% of positive cases. The low percentages of agreement by all four methods may be due to several factors, including sample error due to the patchy nature of infection, virulence of the organism and its urease production, as well as culture and pathological techniques[8,9]. In this study, culture and histology demonstrated a low diagnostic yield compared to the previous report[10]. The success rate for cultivation of *H. pylori* from biopsy sample in our microbiology laboratory was only 37 of 122 samples (30.3%), whereas 93 of 122 samples (76.2%) gave positive results by CLO® test. In addition, positive results were noted in only 35.2% for all histological examinations.

The results of *H. pylori* detection by CLO® test were almost closely identical to those of nested-PCR. However, nested-PCR could detect *H. pylori* in 5 more biopsy samples compared to CLO® test. In three of the four negative PCR cases, the culture and histology results were also negative. Nested-PCR in this study appeared to be more sensitive and more specific than the CLO® test. At present, there is no single test that is as reliable as the "gold standard" for the diagnosis of *H. pylori* infection. Several investigators have suggested that culture of the organism and histological examination directly from gastric biopsy sample should be accepted as "gold standard"[11]. In this study, we did not attempt to name a single test as the çgold standardé, but determined by modifying from the previous reports[6,7]. Although, CLO® test is considered rapid, economical and is widely used in clinical practice, this test may give significant false negative results. Nested-PCR can be used, therefore, for confirmation of *H. pylori* infection if CLO® test yields a negative result.

In conclusion, this study showed that nested-PCR technique had a high sensitivity, specificity and accuracy for detection of *H. pylori* infection. This tech-

nique may be another useful option as a diagnostic tool in such a limited resources setting as ours.

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