NESTED POLYMERASE CHAIN REACTION FOR DETECTION OF HELICOBACTER PYLORI IN GASTRIC BIOPSY SPECIMENS

Orawan Ottiwet¹, Chariya Chomvarin¹, Kunyaluk Chaicumpar¹, Wises Namwat¹ and Pisaln Mairiang²

¹Department of Microbiology, ²Department of Medicine, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand

Abstract. Sensitivity and specificity are important for tests used to detect Helicobacter pylori infection from gastric biopsy specimens. Molecular methods, such as PCR and nested PCR, are sensitive methods for H. pylori detection. The objective of this study was to evaluate the performance of PCR and nested PCR compared to culture, the rapid urease test (RUT) and histology for the diagnosis of H. pylori in 130 gastric biopsy specimens from symptomatic dyspeptic patients. Sensitivity and specificity with PCR were 91 and 100% and with nested PCR were 95 and 97%, respectively. H. pylori was detected by PCR and nested PCR at levels as low as 125 fg (70 cells) and 25 fg (14 cells), respectively. These results suggest nested PCR is a highly sensitive direct method to detect H. pylori infection from biopsy specimens.

Key words: Helicobacter pylori, nested PCR, gastric biopsy, detection

INTRODUCTION

Helicobacter pylori has been recognized as a major risk factor for the development of gastritis, gastric and duodenal ulcers, and gastric cancer (Dunn et al, 1997). Accurate detection is essential for clinical management, especially for the eradication of the bacteria following treatment (Parsonnet et al, 1994; Dunn et al, 1997). Several techniques are available to diagnose H. pylori infection. These methods have been classified as invasive and non-invasive. Invasive methods include culturing, the rapid urease test (RUT) and histology (Dunn et al, 1997). Non-invasive methods include the urea breath test (UBT), antibody detection and a stool antigen test (Dzierzanowska-Fangrat et al, 2006).

The culture method has been the standard method for detecting this bacterium because it allows selection of appropriate antimicrobial therapy (Krogfelt et al, 2005), but it is time consuming, expensive and difficult to perform since H. pylori is a fastidious growth-requiring microorganism (Chowdhury et al, 1991). RUT and histology are also widely used to diagnose H. pylori infection, but they can give false positive results because of the presence of other urease-producing bacteria that have similar morphology (Khanolkar-Gaitonde et al, 2000). Among non-invasive methods,
the UBT is recognized as being an excellent method; however, it is theoretically subject to false positives from urease-positive bacteria present in the oral cavity and it is expensive to perform (Krogfelt et al., 2005). Specific antibody detection methods, such as ELISA, may not be appropriate for countries like Thailand where this bacterium is endemic (Deankanob et al., 2006). Thus, there is no single best method for the diagnosis of *H. pylori* because of variations in sensitivity, running time and reproducibility among the techniques (Kisa et al., 2002).

Molecular methods, such as PCR and nested PCR have been developed for detection of various microorganisms, including *H. pylori*, because they can detect small numbers of organisms and nucleic acids in clinical specimens (Li et al., 1996; Kisa et al., 2002; Singh et al., 2008). Nested PCR has been reported to be more sensitive than PCR. These methods, however, vary in sensitivity and specificity, depending on the target genes used (Krogfelt et al., 2005; Singh et al., 2008; Fonseca et al., 2009). In the present study, the performance of PCR and nested PCR were evaluated and compared to culture, RUT and histology for the diagnosis of *H. pylori* from gastric biopsy specimens in symptomatic dyspeptic patients. PCR and nested PCR can be used with the same biopsy samples employed for both commercial and in-house RUT, resulting in a reduction in the number of specimens required to be taken.

**MATERIALS AND METHODS**

**Patients and gastric biopsy specimens**

Samples were collected from dyspeptic patients attending the endoscopy unit of Srinagarind Hospital, Khon Kaen, Thailand. Patients taking antibiotics or omeprazole within 2 weeks before sampling were excluded. One hundred thirty specimens were collected from patients with various dyspeptic symptoms, including 98 patients with gastritis (GT), 12 with peptic ulcer disease (PUD), 18 with gastric cancer (GCA) and 2 with other diseases. Biopsy specimens from each patient were taken from the gastric antrum and corpus for bacterial culture, RUT and histology. After completing the RUT, gastric biopsy samples were isolated from agar of the RUT assay and stored at -20°C in phosphate-buffered saline (PBS) until DNA extraction was performed.

The study was approved by the Khon Kaen University Ethical Committee for Human Research and each subject gave informed consent before enrolling in the study.

**Culture method**

Isolation and identification of *H. pylori* were performed as described elsewhere (Chomvarin et al., 2008). In brief, gastric biopsy specimens were processed by grinding with 200 µl of normal saline solution (NSS). Suspensions were cultured on 7% human blood containing the antibiotic supplement SR 147 under microaerophilic conditions at 37°C. Plates were examined after 4 and 7 days of incubation. *H. pylori* was identified by its spiral shape on Gram’s staining and being positive on the urease, oxidase and catalase tests.

**Commercial rapid urease test (cRUT)**

The antrum and corpus biopsy specimens were immediately placed in RUT media (Pronto Dry test, Medical Instrument Corporation, Switzerland) and the test performed according to the manufacturer’s instructions. A positive RUT was indicated when the color changed from yellow to pink.
**NESTED PCR FOR H. PYLORI DETECTION**

**In-house rapid urease test (iRUT)**

The iRUT was performed as described previously (Chomvarin et al, 2006). Positive results were the same as in the (cRUT).

**Histological examination**

One each of the antrum and corpus biopsies were fixed in 10% buffered formalin before being embedded in paraffin. Four sections 3-4 μ thick were stained with modified Warthin-Starry stain. A positive result was when spiral organisms were found on the mucosal surface.

**DNA extraction**

DNA extraction was performed according to the manufacturer’s instructions using a genomic DNA purification kit (Puregene DNA purification system, Gentra system, Minneapolis, MN). In brief, gastric biopsy samples obtained from RUT agar were incubated with 450 μl of cell lysis solution and 2.5 μl of proteinase K solution for 3 hours at 55ºC. The lysate then was incubated at 98ºC for 10 minutes and 2.5 μl of RNase A solution were added and incubated at 37ºC for 60 minutes. A 200 μl aliquot of protein precipitation solution was added and the solution was centrifuged at 13,000 g for 3 minutes. The supernatant was collected and 400 μl of 100% isopropanol was added and the solution was centrifuged at 13,000g for 5 minutes. The pellet was washed with 300 μl of 70% ethanol and dissolved in 50 μl DNA hydration solution by incubating for 1 hour at 65ºC. DNA was stored at -20ºC until used.

**PCR detection of glm**

Specific primers designed were based on Lu et al (1999); the size of the PCR amplicon is shown in Table 1. PCR was performed in a final volume of 50 μl containing 10x reaction buffer, 400 μM of each dNTP, 2 mM MgCl₂, 25 pM of each primer, 1.25 U of Taq polymerase and 400 ng of target DNA. The thermal cycler (Perkin-Elmer, Gene Amp, PCR 2400) was programmed for 35 amplification cycles consisting of 93ºC for 1 minute, 55ºC for 1 minute, and 72ºC for 1 minute.

**Nested PCR detection of ureA**

Specific inner and outer primers for detection of ureA in this study were designed and modified based on Kisa et al (2002) using the H. pylori urease A sequence (accession no. AF373584). The design and verification of primer sequences was accomplished using the database search program, Blastn (http://www.ncbi.nlm.nih.gov/blast/blast.cgi). PROLIGO-Oligos Parameter Calculation program (http://proligo2.proligo.com/Calculation/calculation_frame_new.html) was used for Tm calculation and Multalin program (http://prodes.toulouse.inra.fr/multalin/multalin.html) for multiple sequence alignment. The primer sequences used are shown in Table 1.

PCR was performed in a final volume of 50 μl containing 10x reaction buffer, 400 μM of each dNTP, 2 mM MgCl₂, 25 pM of each primer, 1.25 U of Taq polymerase and 400 ng of target DNA. The thermal cycler for outer ureA was programmed for 40 amplification cycles, consisting of 94ºC for 1 minute, 62ºC for 30 seconds, and 72ºC for 30 seconds. Then, 1 μl of amplified product was used for the second round of amplification. PCR was performed in a final volume of 50 μl containing 25 pM of each inner primer, the same buffer as in the first round of amplification and 40 amplification cycles of 94ºC for 1 minute, 59ºC for 30 seconds, and 72ºC for 30 seconds.

The specificity of PCR H. pylori detection was examined using Proteus mirabilis, Pseudomonas aeruginosa, Klebsiella pneumoniae, Enterobacter spp, Escherichia coli and human DNA.
Table 1

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer sequence</th>
<th>PCR product (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>glm</td>
<td>F-5’-AAGCTTTTAGGGGTAGGGGTTT-3’</td>
<td>294</td>
<td>(Lu et al, 1999)</td>
</tr>
<tr>
<td></td>
<td>R-5’-AAGCTTACTTCTAAACACTAAGGC-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ureA</td>
<td>F-5’-GCT AAT GGT AAA TTA GTT CCT GG-3’</td>
<td>411</td>
<td>Modified</td>
</tr>
<tr>
<td>outer</td>
<td>R-5’-CTC CTT AAT TGT TTT TAC ATA GTT G-3’</td>
<td></td>
<td>(Kisa et al, 2002)</td>
</tr>
<tr>
<td>inner</td>
<td>F-5’-AGT TCC TGG TGA GTT GTT CTT AA-3’</td>
<td>350</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>R-5’-AAC CAC GCT CTT TAG CTT C-3’</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Analysis of PCR amplicons

Amplicons were separated by 1.5% agarose gel-electrophoresis and visualized by ethidium bromide staining using 50 bp DNA ladders as markers. Positive results had a 294 bp amplicon of glm and 350 and 411 bp amplicons of ureA.

Statistical analysis

Evaluation of diagnostic methods to detect H. pylori in gastric biopsy specimens were evaluated for sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV). The criteria for a true positive H. pylori infection were 1) a positive culture or 2) negative culture but positive RUT and histological examination (Pajares-Garcia, 1998; Liao et al, 2003).

RESULTS

Determination of sensitivity and specificity of PCR and nested PCR

Sensitivities of H. pylori detection by PCR and nested PCR were investigated using a serial dilution of H. pylori genomic DNA. As little as 125 fg or 25 fg of H. pylori template DNA were sufficient to detect glm by PCR or ureA by nested PCR, respectively (Fig 1, 2). Based on the size of the complete genome for H. pylori, calculated by http://www.ncbi.nlm.nih.gov/sites/entrez?cmd=Retrieve&db=Nucleotide&list_uids=297379223&dopt=GenBank&RID=19VKIN1Y015&log$=nucleot&blast_rank=1, 25 fg and 125 fg of H. pylori DNA correspond to approximately 14 and 70 bacterial cells, respectively. To determine the specificity of primers used for PCR detection of H. pylori, 100 ng of genomic DNA from Enterobacter spp, K. pneumoniae, E. coli, P. mirabilis, P. aeruginosa and human leukocytes were used as templates for amplification of glm and ureA by PCR and nested PCR, respectively (data not shown). The DNA of these bacteria and human leukocytes were not amplified by these primers.

Detection of H. pylori

Of the 130 specimens examined by H. pylori culture, RUT, histology, PCR and nested PCR, 49 (38%) were positive on culture, 63 (48%) on RUT, 73 (56%) on histological examination, 57 (44%) on PCR and 63 (48%) on nested PCR. Forty-seven samples (36%) were positive by all 5 diagnostic methods, 8 (6%) by four methods, 4 (3%) by three methods, 4 (3%) by two methods and 18 (14%) by only one method (Table 2). The sensitivities, specificities,
NESTED PCR FOR *H. pylori* DETECTION

When each of the 35 cRUT and iRUT biopsy specimens for *H. pylori* detection were compared to PCR and nested PCR, the results were the same, except one iRUT produced a color change at nearly 24 hours, but gave negative results on both PCR and nested PCR.

**DISCUSSION**

In this study, five different diagnostic methods (culture, RUT, histology, PCR and nested PCR) were used to detect *H. pylori* infection in 130 gastric biopsy specimens. PCR and nested PCR were performed on the same biopsy samples that were subjected to RUT, resulting in a reduction in the number of biopsies at endoscopy, avoiding the bias of the distribution of the organisms in the stomach. Positive criteria for *H. pylori* infection were having a positive culture or at least 2 of the 5 methods positive. Nested PCR was the method with the highest sensitivity (95%) and good specificity (97%) of the tested methods, since it can detect a lower number of nonviable and viable cells than other methods (Clayton *et al.*, 1992) and a false positive result on nested PCR probably reflects a real positive result because of its high sensitivity. There were only 2 false negative samples (1.5%) which might be explained by improper handing of the biopsy sample, such as prolonged transportation, storage at room temperature or cell

**Fig 1**–Sensitivity of *glm* PCR for detection of *H. pylori* DNA. The expected amplicon is 294 bp. Lanes 1-11, *H. pylori* DNA; lane 12, negative control; lane M, DNA size markers.

**Fig 2**–Sensitivity of *ureA* nested PCR for detection of *H. pylori* DNA. The expected amplicons are 350 and 411 bp. Lanes 1-13, *H. pylori* DNA; lane 14, negative control; lane M, DNA size markers.
Table 2
Comparison of *H. pylori* infection detected by five diagnostic methods.

<table>
<thead>
<tr>
<th>Diagnostic method</th>
<th>Total number</th>
<th>No. of infected specimens (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RUT</td>
<td>Culture</td>
<td>Histology</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Total</td>
<td>130</td>
<td>63 (49)</td>
</tr>
</tbody>
</table>

*a* Infection was considered positive when there was a positive culture or when at least two of the five methods were positive.

Table 3
Sensitivity, specificity, positive and negative predictive values of culture, RUT, histological examination, PCR and nested PCR on 130 gastric biopsy specimens.

<table>
<thead>
<tr>
<th>Method</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture</td>
<td>77</td>
<td>100</td>
<td>100</td>
<td>81</td>
</tr>
<tr>
<td>RUT</td>
<td>94</td>
<td>96</td>
<td>95</td>
<td>94</td>
</tr>
<tr>
<td>Histology</td>
<td>94</td>
<td>83</td>
<td>85</td>
<td>93</td>
</tr>
<tr>
<td>PCR</td>
<td>91</td>
<td>100</td>
<td>100</td>
<td>92</td>
</tr>
<tr>
<td>Nested PCR</td>
<td>95</td>
<td>97</td>
<td>97</td>
<td>97</td>
</tr>
</tbody>
</table>

PPV, Positive predictive value; NPV, Negative predictive value

.....
NESTED PCR FOR H. PYLORI DETECTION

Table 4
Comparison of positive criteria, PCR and nested PCR methods for detecting H. pylori in patients with different clinical outcomes.

<table>
<thead>
<tr>
<th>Method</th>
<th>GT N=98</th>
<th>PU N=12</th>
<th>GCA N=18</th>
<th>Other N=2</th>
<th>Total samples (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive criteriaa</td>
<td>46 (47)</td>
<td>7 (58)</td>
<td>9 (50)</td>
<td>1 (50)</td>
<td>63 (48.5)</td>
</tr>
<tr>
<td>PCR</td>
<td>43 (43)</td>
<td>6 (50)</td>
<td>7 (39)</td>
<td>1 (50)</td>
<td>57 (44)</td>
</tr>
<tr>
<td>Nested PCR</td>
<td>48 (49)</td>
<td>7 (58)</td>
<td>7 (39)</td>
<td>1 (50)</td>
<td>63 (48.5)</td>
</tr>
</tbody>
</table>

aPositive criteria were considered as a culture positive or at least two methods positive. GT, gastritis; PU, peptic ulcer; GCA, gastric cancer; other, gastroesophageal reflux (1) and duodenitis (1).

many factors, such as the absence of H. pylori in specimens, the target genes used, irregular distribution of H. pylori in the gastric mucosa and improper transport conditions (Lu et al, 1999; Fonseca et al, 2009).

The false positives with RUT (3 samples) may be explained by contamination from other urease positive organisms in the gastric biopsy specimens due to the passage of the endoscope into the stomach (Smith et al, 2004). The lower sensitivity of the culture method, compared to the other 4 methods, may be explained by such factors as the low number of microorganisms, the loss of viability of the organisms during the transport (Kisa et al, 2002; Brooks et al, 2004), and the coccoid form of this microorganism (Hammar et al, 1992; Kisa et al, 2002). In the present study, RUT had a higher sensitivity than the culture, histology and PCR methods, and was nearly equal in the sensitivity and specificity to the nested PCR. This suggests RUT is a good technique for detecting H. pylori, and the sample can be used for further investigations of molecular epidemiology and detection of resistant strains from the same biopsies (Cirak et al, 2007; Kulsuntiwong et al, 2008).

Previous studies have found that 10-100 H. pylori cells can be detected from clinical specimens (Brooks et al, 2004) consistent with our findings. Besides the detection of H. pylori on biopsy specimens, PCR may be used for molecular epidemiological studies, virulence gene markers and drug resistance evaluations (Megraud and Lehours, 2007; Chomvarin et al, 2008; Monteiro et al, 2009). The high sensitivity and specificity of nested PCR may be applied to samples from non-invasive methods of detecting H. pylori, such as stool and saliva, or may be used with environmental samples.

In summary, nested PCR can be used for the diagnosis of H. pylori on clinical specimens because it is highly sensitive, specific and can be used on biopsy samples from RUT.

ACKNOWLEDGEMENTS

This study was supported by a research grant from the Faculty of Medicine, Khon Kaen University, Thailand. We would like to thank the staff of the Endoscopy Unit for their help with specimen collection.

Vol 41 No. 6 November 2010 1429
REFERENCES


Parsonnet J, Hansen S, Rodriguez L, et al. 1430 Vol 41 No. 6 November 2010

