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

Orawan Ottiwet[1], Chariya Chomvarin[1], Kunyaluk Chaicumpar[1], Wises Namwat[1] and Pisaln Mairiang[2]

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

Abstract. Sensitivity and specificity are important for tests used to defect *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,

Correspondence: Chariya Chomvarin, Department of Microbiology, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand.
Tel: 66 (0) 43 363808; Fax: 66 (0) 43 348385
E-mail: chariya@kku.ac.th

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 symptomps, 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 -20ºC 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 37ºC. 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.

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 13,000*g* 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, 200 µM of each dNTP, 1.5 mM $MgCl_2$, 0.2 µM 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 $MgCl_2$, 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
Primers used and amplicon sizes for detection of *glm* and *ureA*.

Target gene	Primer sequence	PCR product (bp)	Reference
glm	F-5′-AAGCTTTTAGGGGTGTAGGGGTTT-3′ R- 5′-AAGCTTACTTTCTAACACTAACGC-3′	294	(Lu *et al*, 1999)
ureA			
outer	F-5′-GCT AAT GGT AAA TTA GTT CCT GG-3′ R-5′-CTC CTT AAT TGT TTT TAC ATA GTT G-3′	411	Modified (Kisa *et al*, 2002)
inner	F-5′-AGT TCC TGG TGA GTT GTT CTT AA-3′ R-5′-AAC CAC GCT CTT TAG CTC TGT C-3′	350	This study

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 *ure*A 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=19VKJN1Y015&log$=nucltop&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,

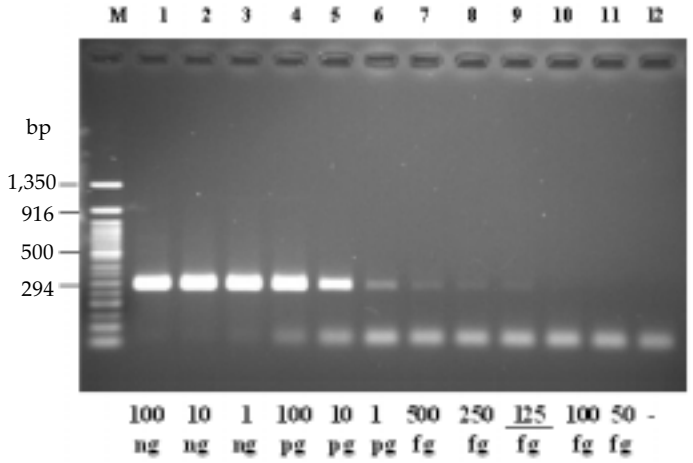

Fig 1–Sensitivity of *glm* PCR for detection of *H. pylori* DNA. The expected amplicon is 294 bp. Lanes 1-11, *H. pylori* DNA; lane 12, negative control; lane M, DNA size markers.

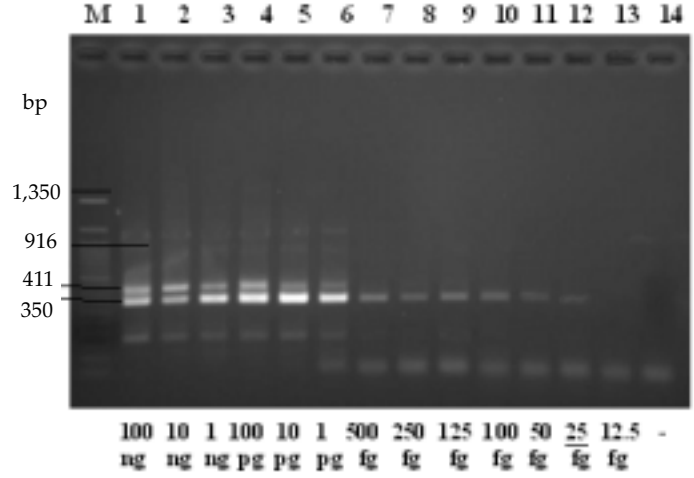

Fig 2–Sensitivity of *ureA* nested PCR for detection of *H. pylori* DNA. The expected amplicons are 350 and 411 bp. Lanes 1-13, *H. pylori* DNA; lane 14, negative control; lane M, DNA size markers.

PPV and NPV show the nested PCR was the most sensitive method and PCR was the most specific method (Table 3). Comparison of positive PCR or nested PCR for detection of *H. pylori* and different clinical outcomes revealed no significant differences between the methods and clinical outcomes (Table 4). 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

Table 2
Comparison of *H. pylori* infection detected by five diagnostic methods.

Diagnostic method					Total number	No. of infected specimens[a] (%)
RUT	Culture	Histology	PCR	Nested PCR		
+	+	+	+	+	47	47 (36)
+	-	+	+	+	6	6 (5)
+	+	-	+	+	2	2 (1.5)
+	-	+	-	+	2	2 (1.5)
+	-	-	+	+	1	1 (1)
-	-	+	+	+	1	1 (1)
+	-	+	-	-	2	2 (1.5)
-	-	+	-	+	2	2 (1.5)
+	-	-	-	-	3	0 (0)
-	-	+	-	-	13	0 (0)
-	-	-	-	+	2	0 (0)
-	-	-	-	-	49	0 (0)
Total					130	63 (49)

[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.

Method	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Culture	77	100	100	81
RUT	94	96	95	94
Histology	94	83	85	93
PCR	91	100	100	92
Nested PCR	95	97	97	97

PPV, Positive predictive value; NPV, Negative predictive value

lysis in RUT medium, resulting in degradation of *H. pylori* DNA (Thoreson *et al*, 1999).

Although Kisa *et al* (2000) found a sensitivity and specificity with nested PCR for *ureA* of 100%, in this study, when the same primers were used, the specificity was lower because of non-specific amplicons from other bacteria and human leukocyte genomes, which had the same amplicon size as *ureA*. Therefore, a new reverse inner primer and modified primers were designed for this study. Fonseca *et al* (2009) found the sensitivity and specificity of *ureA* PCR were 93.3% and 95.8%, respectively, which were lower than our results of 95% and 97%, respectively. Differences in sensitivity and specificity depend on

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

Method	No. of positive samples (%)				
	GT *N*=98	PU *N*=12	GCA *N*=18	Other *N*=2	Total samples (%)
Positive criteria[a]	46 (47)	7 (58)	9 (50)	1 (50)	63 (48.5)
PCR	43 (43)	6 (50)	7 (39)	1 (50)	57 (44)
Nested PCR	48 (49)	7 (58)	7 (39)	1 (50)	63 (48.5)

[a]Positive 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.

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REFERENCES

Brooks HJ, Ahmed D, McConnell MA, *et al.* Diagnosis of *Helicobacter pylori* infection by polymerase chain reaction: is it worth it? *Diagn Microbiol Infect Dis* 2004; 50: 1-5.

Chomvarin C, Chantarasuk Y, Mairiang P, *et al.* Sensitivity and specificity of an in-house rapid urease test for detecting *Helicobacter pylori* infect in gastric biopsy. *Southeast Asian J Trop Med Public Health* 2006; 37: 312-9.

Chomvarin C, Namwat W, Chaicumpar K, *et al.* Prevalence of *Helicobacter pylori vacA*, *cag*A, *cag*E, *ice*A and *bab*A2 genotypes in Thai dyspeptic patients. *Int J Infect Dis* 2008; 12: 30-6.

Chowdhury MN, al-Hedai-Thy SS, Mofleh IA, *et al.* Use of selective and non-selective media for the isolation of *Helicobacter pylori*. *Trop Gastroenterol* 1991; 12: 73-6.

Cirak MY, Akyon Y, Megraud F. Diagnosis of *Helicobacter pylori*. *Helicobacter* 2007; 12 (suppl 1): 4-9.

Clayton CL, Kleanthous H, Coates PJ, *et al.* Sensitive detection of *Helicobacter pylori* by using polymerase chain reaction. *J Clin Microbiol* 1992; 30: 192-200.

Deankanob W, Chomvarin C, Hahnvajanawong C, *et al.* Enzyme-linked immunosorbent assay for serodiagnosis of *Helicobacter pylori* in dyspeptic patient and volunteer blood donors. *Southeast Asian J Trop Med Pulbic Health* 2006; 37: 958-65.

Dunn BE, Cohen H, Blaser MJ. *Helicobacter pylori*. *Clin Microbiol Rev* 1997; 10: 720-41.

Dzierzanowska-Fangrat K, Lehours P, Megraud F, *et al.* Diagnosis of *Helicobacter pylori* infection. *Helicobacter* 2006; 11 (suppl 1): 6-13.

Fonseca TL, Moraes EP, Juliano CR, *et al.* Detection of *Helicobacter pylori* by phenotypic and genotypic methods. *Dig Dis Sci* 2010; 55: 1643-8.

Hammar M, Tyszkiewicz T, Wadstrom T, *et al.* Rapid detection of *Helicobacter pylori* in gastric biopsy material by polymerase chain reaction. *J Clin Microbiol* 1992; 30: 54-8.

Khanolkar-Gaitone SS, Reubish GK, Jr, Lee CK, *et al.* Isolation of bacteria other than *Helicobacter pylori* from stomachs of squirrel monkeys (Saimiri spp) with gastritis. *Dig Dis Sci* 2000; 45: 272-80.

Kisa O, Albay A, Mas MR, *et al.* The evaluation of diagnostic methods for the detection of *Helicobacter pylori* in gastric biopsy specimens. *Diagn Microbiol Infect Dis* 2002; 43: 251-5.

Krogfelt KA, Lehours P, Megraud F. Diagnosis of *Helicobacter pylori* infection. *Helicobacter* 2005; 10 (suppl 1): 5-13.

Kulsuntiwong P, Chomvarin C, Chaicumpar K, *et al.* Antimicrobial susceptibility of *Helicobacter pylori* isolated from gastric biopsies in dyspeptic patients. *Southeast Asian J Trop Med Public Health* 2008; 39: 1102-9.

Li C, Ha T, Ferguson DA, Jr, *et al.* A newly developed PCR assay of *H. pylori* in gastric biopsy, saliva, and feces. Evidence of high prevalence of *H. pylori* in saliva supports oral transmission. *Dig Dis Sci* 1996; 41: 2142-9.

Liao CC, Lee CL, Lai YC, *et al.* Accuracy of three diagnostic tests used alone and in combination for detecting *Helicobacter pylori* infection in patients with bleeding gastric ulcers. *Chin Med J (Engl)* 2003; 116: 1821-6.

Lu JJ, Perng CL, Shyu RY, *et al.* Comparison of five PCR methods for detection of *Helicobacter pylori* DNA in gastric tissues. *J Clin Microbiol* 1999; 37: 772-4.

Megraud F, Lehours P. *Helicobacter pylori* detection and antimicrobial susceptibility testing. *Clin Microbiol Rev* 2007; 20: 280-322.

Monteiro L, Oleastro M, Lehours P, *et al.* Diagnosis of *Helicobacter pylori* infection. *Helicobacter* 2009; 14 (suppl 1): 8-14.

Pajares-Garcia JM. Diagnosis of *Helicobacter pylori*: invasive methods. *Ital J Gastroenterol Hepatol* 1998; 30 (suppl 3): S320-3.

Parsonnet J, Hansen S, Rodriguez L, *et al.*

Helicobacter pylori infection and gastric lymphoma. *N Engl J Med* 1994; 330: 1267-71.

Singh V, Mishra S, Rao GR, *et al.* Evaluation of nested PCR in detection of *Helicobacter pylori* targeting a highly conserved gene: HSP60. *Helicobacter* 2008; 13: 30-4.

Smith SI, Oyedeji KS, Arigbabu AO, *et al.* Comparison of three PCR methods for detection of *Helicobacter pylori* DNA and detection of *cagA* gene in gastric biopsy specimens. *World J Gastroenterol* 2004; 10: 1958-60.

Thoreson AC, Borre M, Andersen LP, *et al. Helicobacter pylori* detection in human biopsies: a comparative PCR assay with intenal control reveals false results. *FEMS Immunol Med Microbiol* 1999; 24: 201-8.