DETECTION OF *HELICOBACTER PYLORI* AND VIRULENCE-ASSOCIATED GENES IN SALIVA SAMPLES OF ASYMPTOMATIC PERSONS IN NORTHEAST THAILAND

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**Abstract.** The aims of the study were to develop nested-PCR (targeting *vacA* and *cagA*), SYBR green quantitative PCR (targeting 16S rDNA) tests and compared them with indirect fluorescent-monoclonal antibody (IFA) method for determination of the prevalence of *Helicobacter pylori* in 118 saliva samples from asymptomatic individuals in Khon Kaen, Thailand. Detection limit of both PCR-based assays was one cell. Prevalence of *H. pylori* in saliva samples was 55% based on the criterion of positivity of IFA test and one of the PCR-based methods or positivity of both PCR assays. Forty-nine percent of *H. pylori* detected carried *cagA*, encoding a cytotoxin associated with severe clinical outcomes. These results imply that the mouth may be an important reservoir for *H. pylori*, with nearly 50% of the virulent type that could possibly lead to gastroduodenal disease.

**Keywords:** *Helicobacter pylori*, *cagA*, indirect fluorescent-monoclonal antibody test, nested-PCR, SYBR green quantitative PCR, saliva, asymptomatic person

**INTRODUCTION**

*Helicobacter pylori*, a gram-negative spiral microaerophilic bacterium, is a causative agent of gastric and duodenal ulcers and gastric cancer (Goodwin *et al*, 1997). The bacterium is found worldwide and exhibit wide geographical variation in prevalence (Mishra *et al*, 2008a). Most infections, however, are asymptomatic (Goodwin *et al*, 1997).

Transmission of *H. pylori* is via oral-oral or fecal-oral route (Silva *et al*, 2010a; Momtaz *et al*, 2012). Many evidences indicate that *H. pylori* can also be transmitted via saliva and dental plaques (Fernandez-Tilapa *et al*, 2011; Momtaz *et al*, 2012). The oral cavity might be a reservoir of *H. pylori*, providing an important habitat from which infection or re-infection of the stomach can occur (Burgers *et al*, 2008; Silva *et al*, 2010b; Al Sayed *et al*, 2014). A high similarity in genotypes of *H. pylori* isolates from saliva, stomach and stool...
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has been demonstrated (Silva et al, 2010a; Momtaz et al, 2012). Thus, it is important to investigate the epidemiology of H. pylori in the oral cavity, which may indicate the risk of H. pylori infection in the stomach and duodenum.

Currently, the standard method for phenotypic identification is culturing of the organism, which takes several days, thus limiting its usefulness for rapid detection of H. pylori infection (Kabir, 2004), nor is it possible to detect the coccoid form or “viable but non-culturable” (VBNC) state, which occurs under stress conditions (Andersen and Rasmussen, 2009). In addition, almost all studies have failed to culture this microorganism from saliva, dental plaque and feces (Luman et al, 1996; Ndip et al, 2003).

PCR-based methods, such as conventional PCR, nested PCR and real-time PCR, have been developed for detection of several microorganisms in clinical and environmental samples (He et al, 2002; Mishra et al, 2008b; Senachai et al, 2013). PCR-based detection of H. pylori has focused on 16S rDNA (Kabir, 2004; Rinttila et al, 2004; Diouf et al, 2009) and vacuolating cytotoxin gene A (vacA) (Chomvarin et al, 2008; Martinez-Carrillo et al, 2014). The vacA is present in all H. pylori strains and can induce formation of intracellular vacuoles, leading to epithelial damage in eukaryotic cells (Reyrat et al, 2000; Palframan et al, 2012). In addition, cytotoxin associated gene A (cagA) is considered a signature genetic marker of H. pylori (Argent et al, 2008). Strains carrying cagA are more virulent than those without this gene (Argent et al, 2008).

Serological tests, such as the indirect fluorescent antibody test (IFA), have been used for detection of such bacteria, such as Vibrio cholerae, in environmental samples using specific antibodies (Hasan et al, 1994). This method allows enumeration of both culturable and non-culturable cells and both the spiral and coccoid forms of H. pylori (Cao et al, 1997). However, IFA is unable to detect the presence of virulence marker genes, such as vacA and cagA.

In Thailand, H. pylori has been reported only in patients with gastro-hepatobiliary disease (Chomvarin et al, 2008; Boonyanugomol et al, 2012a), but has not been investigated asymptomatic individuals. Thus, the present study determined the prevalence of H. pylori in saliva of asymptomatic persons in Khon Kaen, northeastern Thailand using nested and quantitative (q)PCR techniques and IFA.

MATERIALS AND METHODS

Bacterial strains

Bacterial strains used were obtained from the Department of Medical Sciences culture collection, Thailand (DMST), American type culture collection (ATCC), and clinical and environmental sources, Srinagarind Hospital and the Department of Microbiology Laboratory, Faculty of Medicine, Khon Kaen University, Thailand.

Asymptomatic subjects

Saliva was collected from 118 randomly selected asymptomatic individuals residing in Khon Kaen Province, Thailand. Inclusion criteria were (i) age between 18 - 80 years, (ii) no previous diagnosis of gastric carcinoma, gastritis or other gastro-hepatobiliary diseases, severe alcohol abuse and drug addiction, and (iii) no consumption of proton-pump inhibitor (PPI), bismuth-containing compounds and antibiotics within the previous 4 weeks.

All subjects signed an informed consent form before saliva sample collection.
The study was approved by the Institutional Human Ethics Committee of Khon Kaen University (HE561444).

**Collection and processing of saliva samples**

Saliva collection was performed according to Silva *et al.* (2009) with slight modification. Approximately 2-3 ml aliquot of salivary flow was collected from each subject into a sterile container. A 2-ml aliquot of saliva was added to 8 ml of Brucella broth (Criterion, Santa Maria, CA) and after 3 and 7 days of enrichment in the broth, 1 ml aliquot of solution was used for nested PCR and real-time PCR assay and another 1 ml for the IFA test. Samples were regarded as *H. pylori*-positive if they showed a positive result by IFA and at least one of the molecular tests (nested PCR/SYBR green real-time PCR) or both molecular methods (nested PCR and real-time PCR) were positive.

**Culturing of Helicobacter pylori**

*H. pylori* strains from bacterial stock cultures were grown on Blood agar (BD Difco, Franklin, NJ) containing 5% whole human blood, 10% bovine serum (Invitrogen, Carlsbad, CA) and 40 mg/ml of 2,3,5-triphenyltetrazolium chloride (Sigma-Aldrich, St Louis, MO) overnight at 37°C under microaerobic condition (5% O₂, 10% CO₂ and 85% N₂) and were examined after 3 and 7 days of incubation. Characteristic colonies of *H. pylori* were confirmed by Gram staining and by oxidase, catalase and urease tests.

**Nested-PCR assay of H. pylori vacA and cagA**

An aliquot saliva solution (1 ml) was centrifuged at 13,000g for 10 minutes and DNA was extracted from pellet using a commercial DNA extraction kit (Qiagen, Hilden, Germany). Primers targeting *H. pylori* vacA were designed using Premier Primer 3.0 (Premier Biosoft International, Palo Alto, CA) and tested for specificity using BLAST software (http://blast.ncbi.nlm.nih.gov/Blast.cgi) from sequence of GenBank accession no. AF191639.1; and targeting cagA from those reported by Huang *et al.* (2009) (Table 1). Specificity of the primers was also evaluated by PCR assay using 100 ng of DNA of reference strains. The amplification reaction of 25 μl contained 100 ng of DNA,1X PCR buffer (RBC Bioscience, Taipei, Taiwan), 0.2 mM each dNTP (Amresco, Cleveland, OH), 50 mM KCl, 0.1 mg/ml BSA, 10 mM (NH₄)₂SO₄, 1.5 mM MgCl₂, 0.2 μM cagA primers (both outer and inner primers) or 0.4 μM vacA primers (both outer and inner primers), and 0.5 U Taq DNA polymerase (RBC Bioscience). A 3 μl aliquot of 1st round PCR was used in the 2nd round of PCR. Thermocycling conducted in a Bio-Rad C1000 thermal cycler (Bio-Rad, Hercules, CA) are described in Table 1. Amplicons (Table 1) were analyzed by 1.5% agarose gel-electrophoresis and visualized under an UV illuminator following ethidium bromide staining.

To optimize the PCR condition of spiked-saliva samples with *H. pylori* or saliva samples collected from subjects, DNA was extracted from 1.0 ml of each sample using commercial DNA extraction kit (QIAGEN) and 500 ng was used as template for the nested PCR and real-time PCR assay.

**qPCR assay of H. pylori 16S rDNA**

SYBR Green qPCR assay was conducted in a 20-μl reaction volume containing 10 μl of 2X SsoAdvanced™ SYBR® Green Supermix (Bio-Rad, Hercules, CA), 1 μM 16S rDNA primers (Table 1) and 100 ng of DNA template of pure *H. pylori* culture or 500 ng of DNA template of saliva samples. Nuclease-free water was used in negative control. Thermocycling were conducted in a CFX96™ Real-time
Table 1. Primer sequences and PCR conditions used for detection of *H. pylori* by nested-PCR and SYBR green quantitative (q) PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequences (5'-3')</th>
<th>Product size (bp)</th>
<th>PCR conditions</th>
<th>Reference</th>
</tr>
</thead>
</table>
| *vacA* (nested PCR) | F-outer: GCATGATTTTGGCACCATTG  
R-outer: TTTTCATATTTAGGGGCAA  
F-inner: GCATGATTTTGGCACCATTG  
R-inner: ATCGCATTGCTCAAGCTCAA | 429  
276 | 95°C 30 s, 54°C 30 s, 72°C 45 s (35 cycles)  
95°C 30 s, 62°C 30 s, 72°C 60 s (35 cycles) | This study  
Huang *et al.*, 2009  
Rinttila *et al.*, 2004 |
| *cagA* (nested-PCR) | F-outer: ACGATTGGAACGCCACC  
R-outer: CGCCATTTGTAACGCCTA  
F-inner: ATAATGCTAAATTAGACAACTTGAGGA  
R-inner: TTAGAATAATCAACAAACATCACGCCAT | 588  
297 | 94°C 60 s, 54°C 60 s, 72°C 60 s (35 cycles)  
94°C 60 s, 60°C 60 s, 72°C 60 s (30 cycles) |  
Indirect fluorescence antibody (IFA) for detecting *H. pylori*

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Fig 1–Specificity of vacA outer (A) and inner (B) primers for detection of genomic DNA of H. pylori and other bacteria using nested-PCR. Lane M, 1 kbp DNA size markers; lanes 1-3, DNA templates of H. pylori DMST 20165, DMST 20979, and one-clinical strain, respectively; lanes 4–22, three strains of Campylobacter jejuni, Streptococcus pyogenes, Staphylococcus epidermidis, Streptococcus pneumoniae, Micrococcus spp, Enterococcus spp, Salmonella spp, Shigella dysenteriae, Klebsiella spp, Escherichia coli, Enterobacter spp, Aeromonas hydrophila, Acinetobacter spp, Pseudomonas aeruginosa, Proteus spp, V. cholerae O1, V. parahaemolyticus, respectively; lane 23, negative control.

RESULTS

PCR primers specificity and detection limit

The PCR primer sets were H. pylori-specific as tested against three reference H. pylori strains and 19 different bacterial species using nested-PCR (Fig 1) and qPCR (Fig 2A). IFA specificity was demonstrated by positive result only with H. pylori (Fig 3). Detection limit of both nested PCR for vacA and SYBR green qPCR for 16S rDNA was 1 cell/PCR reaction (10 cells/ml).

Detection of H. pylori in saliva samples

The prevalence of H. pylori in saliva samples of 118 asymptomatic persons was 57% by nested-PCR (Fig 1), 56% by SYBR green qPCR (Fig 2B) and 50% by IFA test (Table 2). True positives numbered 65
Fig 2–DNA melting profiles and fluorescence amplification curve of SYBR green quantitative PCR. Amplification conditions are described in Materials and Methods and Table 1. A. DNA melting profiles using 16S rDNA primer set of H. pylori DMST 20165 (open squares); H. pylori DMST 20979 (open triangles), H. pylori clinical strains (open circles), other bacteria (solid lines). B. Fluorescence amplification curves (a) and DNA melting profiles (b) of H. pylori DNA (open circles) from saliva of an asymptomatic subject. Closed circles, H. pylori control; closed squares, non-H. pylori control. RFU, relative fluorescence unit.

Fig 3–Fluorescence photomicrography detection of H. pylori in saliva of asymptomatic individuals. (A) Positive control.(B) Negative control. (C) Positive sample and (D) negative sample.
Fig 4–Agarose gel-electrophoresis of *H. pylori* vacA (A) and cagA (B) amplicons using nested-PCR. Amplification conditions are described in Materials and Methods and Table 1. Lane M, 1 kbp DNA size markers; lane 1, positive control; lane 2, negative control; lanes 3-8, saliva samples of asymptomatic subjects.

(55%) according to the criterion of positive by IFA test and at least one PCR method or positive by both PCR methods. Of the 65 asymptomatic persons positive for *H. pylori* in saliva samples, 32(49%) were positive for the *H. pylori* cagA gene (Fig 4).

**DISCUSSION**

In this study, nested-PCR and SYBR green qPCR methods for direct detection of *H. pylori* from saliva samples were developed, using vacA and 16S rDNA as target, respectively. Although other studies have employed only primer pairs targeting vacA for detection of *H. pylori*, the primers showed cross-reactions with other organisms leading to false positive results (Sugimoto *et al*, 2009; Singh *et al*, 2012). Thus, we developed a new primer set for vacA that showed more specificity. In addition, 16S rDNA is a highly sensitive and specific target for *H. pylori* detection used by a number of research groups (Rinttila *et al*, 2004; Diouf *et al*, 2009).

Molecular epidemiological studies have focused on detection of vacA and cagA (Arents *et al*, 2001; Chomvarin *et al*, 2008; Boonyanugomol *et al*, 2012b). cagA is an important virulence gene related to severe clinical outcomes in *H. pylori*, such as gastric cancer and gastric mucosal atrophy (Andreson *et al*, 2002; Monstein *et al*, 2010). Our results showed that half of *H. pylori* isolates in saliva samples of asymptomatic individuals harbored cagA. Therefore, these persons might be at risk of gastroduodenal infection leading to associated diseases (Parsonnet *et al*, 1999; Silva *et al*, 2009; Momtaz *et al*, 2012).

The nested-PCR and SYBR green qPCR using pure cultures and spiked-saliva samples could detect 1 cell/PCR assay. By comparison, previous studies reported sensitivity of nested-PCR detection of *H. pylori* in saliva samples equivalent to 1-10^2^ cells (Kabir, 2004). Sensitivity of detection of ureC using SYBR green qPCR was reported as 10^3^ cells (He *et al*, 2002). Five cells of *H. pylori* per PCR assay could be detected using serially diluted DNA when the target was a 26-kDa Helicobacter species-specific antigen gene (Mikula *et al*, 2003). The protocol developed in this study has a higher sensitivity and specificity.
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Table 2

Detection of *H. pylori* in 118 saliva samples by nested-PCR, SYBR green quantitative qPCR and indirect fluorescent antibody (IFA) test.

<table>
<thead>
<tr>
<th>Method</th>
<th>Total number (%)</th>
<th>Evaluation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nested-PCR</td>
<td>qPCR</td>
<td>IFA</td>
</tr>
<tr>
<td>+ + +</td>
<td>23 (19)</td>
<td>TP</td>
</tr>
<tr>
<td>+ + -</td>
<td>22 (19)</td>
<td>TP</td>
</tr>
<tr>
<td>+ - +</td>
<td>9 (8)</td>
<td>TP</td>
</tr>
<tr>
<td>- + +</td>
<td>11 (9)</td>
<td>TP</td>
</tr>
<tr>
<td>- - +</td>
<td>16 (14)</td>
<td>FP</td>
</tr>
<tr>
<td>+ - -</td>
<td>13 (11)</td>
<td>FP</td>
</tr>
<tr>
<td>- + -</td>
<td>10 (8)</td>
<td>FP</td>
</tr>
<tr>
<td>- - -</td>
<td>14 (12)</td>
<td>TN</td>
</tr>
<tr>
<td>Totals</td>
<td>118 (100)</td>
<td>65 (55%)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

TP, true positive; TN, true negative; FP, false positive. <sup>a</sup>Positive according to IFA test and at least one PCR method or by both PCR methods.

The prevalence of *H. pylori* in oral cavity has been reported from 0% to 100% (Silva *et al*, 2010b). For example, *H. pylori* was detected in 75% of saliva samples using a PCR assay (Li *et al*, 1995), 97% of dental plaque samples and 55% of saliva samples of patients with gastrointestinal symptoms using nested-PCR (Song *et al*, 2000), and 45.7% of saliva samples in asymptomatic subjects using nested-PCR (Mishra *et al*, 2008a). There are several hypotheses to explain such differing prevalences: i) variation in sensitivity and specificity of each diagnostic method, ii) variation in subject groups, iii) variation of sample collection methods, iv) low number of *H. pylori* cells in samples, and v) temporal variation in saliva samples (Kabir, 2004).

This is the first report to quantify *H. pylori* cells in saliva of asymptomatic persons in Thailand. On average, $10^2$-$10^3$ cells/ml were detected. Previous studies of *H. pylori* in saliva and/or dental plaque have reported 1-213 cells/mg of sample in adult patients with active *H. pylori* infection, and 6-76 cells/mg in adult patients and 4-94 cells/mg in children without *H. pylori* infection (Song *et al*, 2000).

IFA test was developed for detection of *H. pylori* in saliva samples. The primary antibody used in this work was specific for *H. pylori* and did not cross-react with other tested bacteria. However, the possibility of reactivity of the antibody with other *Helicobacter* spp that we did not test needs to be considered (Hegarty *et al*, 1999). We used two PCR-based methods combined with IFA method for detection of *H. pylori* to enhance specificity. The combination of methods showed that both nested-PCR and qPCR assays are more sensitive for detecting *H. pylori* in saliva samples than IFA test. PCR can detect both live and non-viable bacterial cells. We suggest that two or more methods should be combined for detection of *H. pylori* in saliva samples to prevent the false negative results. Discrepancy between two PCR methods could have occurred because...
i) the primers used were different in two methods, and ii) the target gene sequences in saliva sample genes may have some variation compared to those genes in GenBank database. Thus, the advantage of using different genes to determine *H. pylori* is to confirm true positive results.

In summary, to the best of our knowledge, this is the first report of the prevalence of *H. pylori* from saliva samples of asymptomatic individuals in Thailand, and the first using PCR-based methods as well as IFA test. Over half of the test subjects were positive and nearly half of *H. pylori* detected carried the virulence *cagA*, indicating the presence of highly pathogenic *H. pylori* in a significant proportion of asymptomatic adults in northeastern Thailand. The association of *cagA*-positive *H. pylori* in saliva and gastrointestinal syndromes should be studied so that appropriate control and prevention measures can be implemented.

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