# 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 *vac*A and *cag*A), 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 *cag*A, 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*, *cag*A, 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 (*vac*A) (Chomvarin *et al*, 2008; Martinez-Carrillo *et al*, 2014). The *vac*A 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 (*cag*A) is considered a signature genetic marker of *H. pylori* (Argent *et al*, 2008). Strains carrying *cag*A 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 *vac*A and *cag*A.

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% $O_2$, 10% $CO_2$ and 85% $N_2$) 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 vac*A and *cag*A**

An aliquot saliva solution (1 ml) was centrifuged at 13,000*g* for 10 minutes and DNA was extracted from pellet using a commercial DNA extraction kit (Qiagen, Hilden, Germany). Primers targeting *H. pylori vac*A 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 *cag*A 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 $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 0.2 μM *cag*A primers (both outer and inner primers) or 0.4 μM *vac*A 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.

| Gene | Primer sequence (5'-3') | Product size (bp) | PCR conditions | Reference |
|---|---|---|---|---|
| *vac*A (nested PCR) | | | | |
| F-outer | GCATGATTTTGGCACCATTG | 429 | 95°C 30 s, 54°C 30 s, 72°C 45 s (35 cycles) | This study |
| R-outer | TTTTCATATTTAGGGGGCAAA | | | |
| F-inner | GCATGATTTTGGCACCATTG | 276 | 95°C 30 s, 62°C 30 s, 72°C 45 s (35 cycles) | |
| R-inner | ATCGCATTGCTCAAGCTCAA | | | |
| *cag*A (nested-PCR) | | | | |
| F-outer | ACGATTGGAACGCCACC | 588 | 94°C 60 s, 54°C 60 s, 72°C 60 s (35 cycles) | Huang *et al*, 2009 |
| R-outer | CGCCATTTGTAACGCCTA | | | |
| F-inner | ATAATGCTAAATTAGACAACTTGAGCGA | 297 | 94°C 60 s, 60°C 60 s, 72°C 60 s (30 cycles) | |
| R-inner | TTAGAATAATCAACAAACATCACGCCAT | | | |
| 16S rDNA (qPCR) | | | | |
| F | ATTTCACACCTGACTGACTAT | 139 | 95°C 20 s, 58°C, 30 s, 72°C, 45 s (35 cycles) | Rinttila *et al*, 2004 |
| R | GAAGATAATGACGGTATCTAAC | | | |

System (BioRad) as described in Table 1. A threshold cycle ($C_T$)value >33 is considered negative for 16S rDNA (Fig 2B). Melting curve analysis showed a single amplicon with melting temperature ($T_m$) of 86.0±0.5°C.

**PCR sensitivity determination**

The detection limits for *H. pylori* of nested PCR and SYBR green qPCR for detection of *H. pylori* were determined by preparing 10-fold serial dilutions of two reference strains of *H. pylori* mixed in equal numbers to obtain $10^0$-$10^7$ cfu/ml. DNA from 1 ml of each dilution was extracted using Puregene DNA Purification System (Qiagen) and 3 μl used as template DNA for the nested PCR and qPCR assay. Each experiment was performed in triplet.

To prepare spiked-saliva samples, bacterial suspensions of *H. pylori* at concentration of $10^7$ to $10^0$ cells/ml were added to saliva samples. DNA was extracted from 1.0 ml of each dilution using commercial DNA extraction kit (Qiagen) and 5 μl were used as template for the nested PCR and qPCR assay.

**Indirect fluorescence antibody (IFA) for detecting *H. pylori***

IFA specificity test was performed according to Wang *et al* (2010). In brief, a 30 μl aliquot of $10^5$ CFU/ml of two reference strains of *H. pylori* and other bacteria (*Escherichia coli, Enterobacter* spp, *Shigella* spp, *Salmonella* spp, *Klebsiella* spp, *Proteus* spp, *Vibrio cholerae, V. parahaemolyticus, Aeromonas hydrophila, Proteus aeruginosa, Staphylococcus epidermidis, S. aureus, Streptococcus*

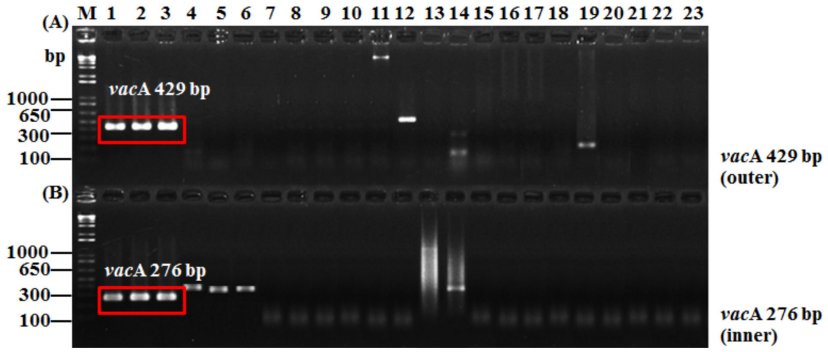


Fig 1–Specificity of *vac*A outer (A) and inner (B) primers for detection of genomic DNA of *H. pylori* and other bacteria using nested-PCR. Lane M, 1 kbp DNA size markers; lanes 1-3, DNA templates of *H. pylori* DMST 20165, DMST 20979, and one-clinical strain, respectively; lanes 4- 22, three strains 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.

*pyogenes*, *Enterobacter faecalis*, *Micrococcus* spp, *Bacillus* spp, *Listeria monocytogenes* and *Candida albicans*) were smeared on microscopic slides and air dried. Slides then were incubated with mouse IgG specific for *H. pylori* (Santa Cruz Biotechnology, Santa Cruz, CA) (1:100 dilution) at 4ºC for 1 hour, then were washed with 0.01 M phosphate-buffered saline (PBS) pH 7.3 and air dried. The 30 µl aliquot of FITC-conjugated goat anti-mouse IgG1 (Dako®, Glostrup, Denmark) (1:200 dilution) was added onto slide and incubated for 1 hour in the dark at room temperature, then slide was washed with 0.01 M PBS pH 7.3 and air dried. Slide was mounted with 1 drop (5 µl) of fluorescent mounting solution (New Horizons Diagnostics, Maryland) prior to observation under a fluorescence microscope (Nikon, Tokyo, Japan).

IFA for the detection of *H. pylori* in saliva was performed as described above. In brief, 1 ml aliquot of saliva suspension was centrifuged at 13,000*g* for 10 minutes and pellet was suspended in 100 µl of PBS. The mixture was added with 12.5 µl of 0.025% yeast extract (Difco, Detroit, MI), 10 µl of 0.002% nalidixic acid (Sigma-Aldrich) and incubated overnight at 25ºC in the dark, followed by addition of 112 µl of 37% formalin. Finally, 30 µl aliquot of the sample was placed on a slide, air dried, incubated with mouse anti-*H. pylori* IgG (1:100 dilution) and processed as described above.

## 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 *vac*A 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

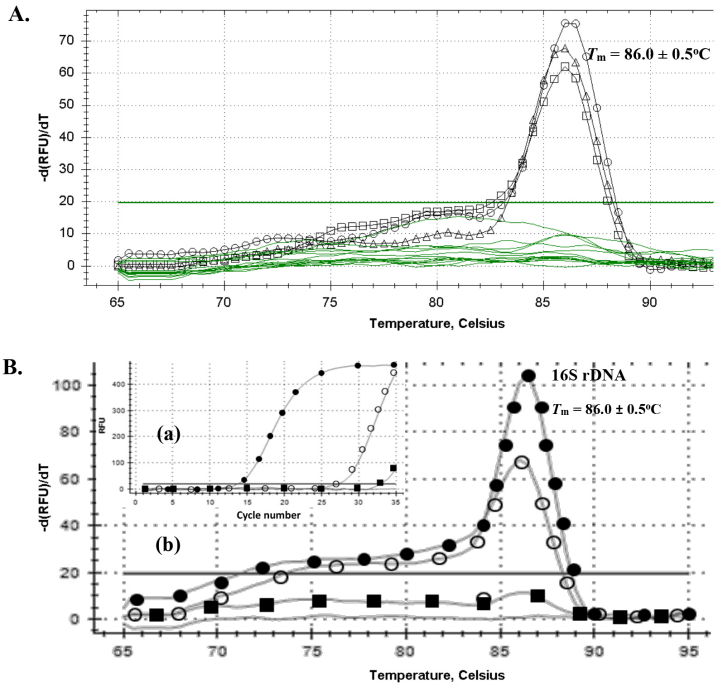


Fig 2–DNA melting profiles and fluorescence amplification curve of SYBR green quantitative PCR. Amplification conditions are described in Materials and Methods and Table 1. A. DNA melting profiles using 16S rDNA primer set of *H. pylori* DMST 20165 (open squares); *H. pylori* DMST 20979 (open triangles), *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.

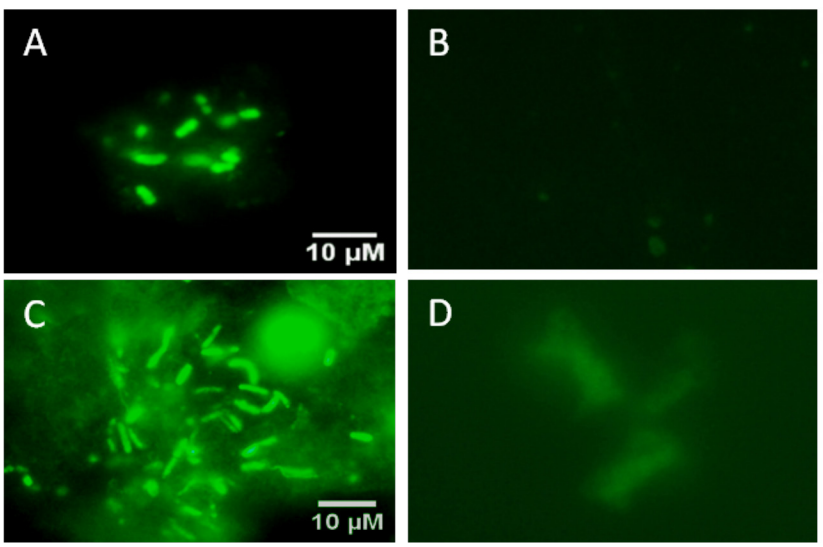


Fig 3–Fluorescence photomicrography detection of *H. pylori* in saliva of asymptomatic individuals. (A) Positive control.(B) Negative control. (C) Positive sample and (D) negative sample.

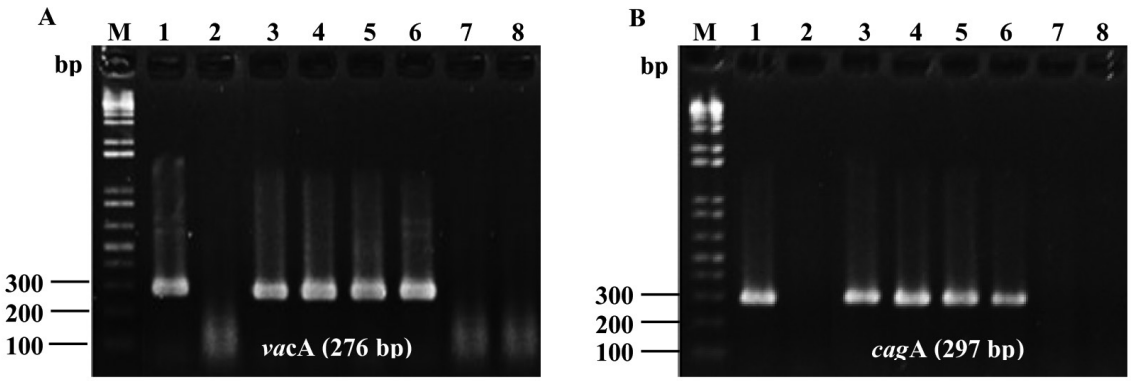


Fig 4–Agarose gel-electrophoresis of *H. pylori vac*A (A) and *cag*A (B) amplicons using nested-PCR. Amplification conditions are described in Materials and Methods and Table 1. Lane M, 1 kbp DNA size markers; lane 1, positive control; lane 2, negative control; lanes 3-8, saliva samples of asymptomatic 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 cag*A 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 *vac*A and 16S rDNA as target, respectively. Although other studies have employed only primer pairs targeting *vac*A 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 *vac*A 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 v*ac*A and *cag*A (Arents *et al*, 2001; Chomvarin *et al*, 2008; Boonyanugomol *et al*, 2012b). *cag*A 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 *cag*A. 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.

Table 2
Detection of *H. pylori* in 118 saliva samples by nested-PCR, SYBR green quantitative qPCR and indirect fluorescent antibody (IFA) test.

| Method | | | Total number (%) | Evaluation |
|---|---|---|---|---|
| Nested-PCR | qPCR | IFA | | |
| + | + | + | 23 (19) | TP |
| + | + | - | 22 (19) | TP |
| + | - | + | 9 (8) | TP |
| - | + | + | 11 (9) | TP |
| - | - | + | 16 (14) | FP |
| + | - | - | 13 (11) | FP |
| - | + | - | 10 (8) | FP |
| - | - | - | 14 (12) | TN |
| Totals | | | 118 (100) | 65 (55%)[a] |

TP, true positive; TN, true negative; FP, false positive. [a]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 *cag*A, indicating the presence of highly pathogenic *H. pylori* in a significant proportion of asymptomatic adults in northeastern Thailand. The association of *cag*A-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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## REFERENCES

Al Sayed A, Anand PS, Kamath KP, *et al.* Oral cavity as an extragastric reservoir of *Helicobacter pylori*. *ISRN Gastroenterol* 2014; 2014: 261369.

Andreson H, Loivukene K, Sillakivi T, *et al.* Association of *cagA* and *vacA* genotypes of *Helicobacter pylori* with gastric diseases in Estonia. *J Clin Microbiol* 2002; 40: 298-300.

Andersen LP, Rasmussen L. *Helicobacter pylori*-coccoid forms and biofilm formation. *FEMS Immunol Med Microbiol* 2009; 56: 112-5.

Arents NL, van Zwet AA, Thijs JC, *et al.* The importance of *vac*A, *cag*A, and *ice*A genotypes of *Helicobacter pylori* infection in peptic ulcer disease and gastroesophageal reflux disease. *Am J Gastroenterol* 2001; 96: 2603-8.

Argent RH, Hale JL, El-Omar EM, Atherton JC. Differences in *Helicobacter pylori* CagA tyrosine phosphorylation motif patterns between western and East Asian strains, and influences on interleukin-8 secretion. *J Med Microbiol* 2008; 57: 1062-7.

Boonyanugomol W, Chomvarin C, Sripa B, *et al.* *Helicobacter pylori* in Thai patients with cholangiocarcinoma and its association with biliary inflammation and proliferation. *HPB (Oxford)* 2012a; 14: 177-84.

Boonyanugomol W, Chomvarin C, Sripa B, *et al.* Molecular analysis of *Helicobacter pylori* virulent-associated genes in hepatobiliary patients. *HPB (Oxford)* 2012b; 14: 754-63.

Burgers R, Schneider-Brachert W, Reischl U, *et al.Helicobacter pylori* in human oral cavity and stomach. *Eur J Oral Sci* 2008; 116: 297-304.

Cao J, Li ZQ, Borch K, Petersson F, Mardh S. Detection of spiral and coccoid forms of *Helicobacter pylori* using a murine monoclonal antibody. *Clin Chim Acta* 1997; 267: 183-96.

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

Diouf A, Martinez-Gomis J, Miquel M, *et al.* Comparison of four different primer sets for detection of *Helicobacter pylori* in gastric biopsies and oral samples by using real-time PCR. *Pathol Biol (Paris)* 2009; 57: 30-5.

Fernandez-Tilapa G, Axinecuilteco-Hilera J, Giono-Cerezo S, *et al. vac*A genotypes in oral cavity and *Helicobacter pylori* seroposi-

tivity among adults without dyspepsia. *Med Oral Patol Oral Cir Bucal* 2011; 16: e175-80.

Goodwin CS, Mendall MM, Northfield TC. *Helicobacter pylori* infection. *Lancet* 1997; 349: 265-9.

Hasan JA, Bernstein D, Huq A, *et al*. Cholera DFA: an improved direct fluorescent monoclonal antibody staining kit for rapid detection and enumeration of *Vibrio cholerae* O1. *FEMS Microbiol Lett* 1994; 120: 143-8.

He Q, Wang JP, Osato M, Lachman LB. Real-time quantitative PCR for detection of *Helicobacter pylori*. *J Clin Microbiol* 2002; 40: 3720-8.

Hegarty JP, Dowd MT, Baker KH. Occurrence of *Helicobacter pylori* in surface water in the United States. *J Appl Microbiol* 1999; 87: 697-701.

Huang Y, Tian XF, Fan XG, Fu CY, Zhu C. The pathological effect of *Helicobacter pylori* infection on liver tissues in mice. *Clin Microbiol Infect* 2009; 15: 843-9.

Kabir S. Detection of *Helicobacter pylori* DNA in feces and saliva by polymerase chain reaction: a review. *Helicobacter* 2004; 9: 115-23.

Li C, Musich PR, Ha T, *et al.* High prevalence of *Helicobacter pylori* in saliva demonstrated by a novel PCR assay. *J Clin Pathol* 1995; 48: 662-6.

Luman W, Alkout AM, Blackwell CC, Weir DM, Plamer KR. *Helicobacter pylori* in the mouth negative isolation from dental plaque and saliva. *Eur J Gastroenterol Hepatol* 1996; 8: 11-4.

Martinez-Carrillo DN, Atrisco-Morales J, Hernandez-Pando R, *et al. Helicobacter pylori vac*A and *cag*A genotype diversity and interferon gamma expression in patients with chronic gastritis and patients with gastric cancer. *Rev Gastroenterol Mex* 2014; 79: 220-8.

Mikula M, Dzwonek A, Jagusztyn-Krynicka K, Ostrowski J. Quantitative detection for low levels of *Helicobacter pylori* infection in experimentally infected mice by real-time PCR. *J Microbiol Methods* 2003; 55: 351-9.

Mishra S, Singh V, Rao GR, *et al.* Prevalence of *Helicobacter pylori* in asymptomatic subjects-a nested PCR based study. *Infect Genet Evol* 2008a; 8: 815-9.

Mishra S, Singh V, Rao GR, *et al*. Detection of *Helicobacter pylori* in stool specimens: comparative evaluation of nested PCR and antigen detection. *J Infect Dev Ctries* 2008b; 2: 206-10.

Momtaz H, Souod N, Dabiri H, Sarshar M. Study of *Helicobacter pylori* genotype status in saliva, dental plaques, stool and gastric biopsy samples. *World J Gastroenterol* 2012; 18: 2105-11.

Monstein HJ, Karlsson A, Ryberg A, Borch K. Application of PCR amplicon sequencing using a single primer pair in PCR amplification to assess variations in *Helicobacter pylori* CagA EPIYA tyrosine phosphorylation motifs. *BMC Res Notes* 2010; 3: 35.

Ndip RN, MacKay WG, Farthing MJ, Weaver LT. Culturing *Helicobacter pylori* from clinical specimens: review of microbiologic methods. *J Pediatr Gastroenterol Nutr* 2003; 36: 616-22.

Palframan SL, Kwok T, Gabriel K. Vacuolating cytotoxin A (VacA), a key toxin for *Helicobacter pylori* pathogenesis. *Front Cell Infect Microbiol* 2012; 2: 92.

Parsonnet J, Shmuely H, Haggerty T. Fecal and oral shedding of *Helicobacter pylori* from healthy infected adults. *JAMA* 1999; 282: 2240-5.

Reyrat JM, Rappuoli R, Telford JL. A structural overview of the Helicobacter cytotoxin. *Int J Med Microbiol* 2000; 290: 375-9.

Rinttila T, Kassinen A, Malinen E, Krogius L, Palva A. Development of an extensive set of 16S rDNA-targeted primers for quantification of pathogenic and indigenous bacteria in faecal samples by real-time PCR. *J Appl Microbiol* 2004; 97: 1166-77.

Senachai P, Chomvarin C, Namwat W, *et al.* Application of tetraplex PCR for detection of *Vibrio cholerae*, *V. parahaemolyticus*,

*V. vulnificus* and *V. mimicus* in cockle. *Southeast Asian J Trop Med Public Health* 2013; 44: 249-58.

Silva DG, Stevens RH, Macedo JM, *et al.* Detection of cytotoxin genotypes of *Helicobacter pylori* in stomach, saliva and dental plaque. *Arch Oral Biol* 2009; 54: 684-8.

Silva DG, Stevens RH, Macedo JM, *et al.* Presence of *Helicobacter pylori* in supragingival dental plaque of individuals with periodontal disease and upper gastric diseases. *Arch Oral Biol* 2010a; 55: 896-901.

Silva DG, Tinoco EM, Rocha GA, *et al.Helicobacter pylori* transiently in the mouth may participate in the transmission of infection. *Mem Inst Oswaldo Cruz* 2010b; 105: 657-60.

Singh A, Hodgson N, Yan M, *et al.* Screening *Helicobacter pylori* genes induced during infection of mouse stomachs. *World J Gastroenterol* 2012; 18: 4323-34.

Song Q, Lange T, Spahr A, Adler G, Bode G. Characteristic distribution pattern of *Helicobacter pylori* in dental plaque and saliva detected with nested PCR. *J Med Microbiol* 2000; 49: 349-53.

Sugimoto M, Wu JY, Abudayyeh S, *et al.* Unreliability of results of PCR detection of *Helicobacter pylori* in clinical or environmental samples. *J Clin Microbiol* 2009; 47: 738-42.

Wang D, Xu X, Deng X, *et al*. Detection of *Vibrio cholerae* O1 and O139 in environmental water samples by an immunofluorescent-aggregation assay. *Appl Environ Microbiol* 2010; 76: 5520-5.