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Contributed Paper

Detection of *Helicobacter pylori* in Aquatic Environments and Drinking Waters in Northeastern Thailand

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ABSTRACT

Helicobacter pylori is a causative agent of gastroduodenal and hepatobiliary diseases. Waterborne transmission of this bacterium has been suggested but has not been demonstrated in Thailand. The aims of this study were to determine the prevalence of *H. pylori* in environmental water and drinking water samples in northeastern Thailand by culture, nested PCR, real-time PCR, reverse transcription (RT)-nested PCR and RT-real-time PCR, and also indirect fluorescent antibody (IFA) assay. We also determined the prevalence of the virulence gene, cytotoxin-associated gene A (*cagA*). The limits of detection of *H. pylori* in pure culture were 6×10^2 CFU/PCR and 1 CFU/PCR according to our newly developed RT-nested PCR and RT-SYBR green qPCR assays, respectively. The overall prevalence of *H. pylori* was 48% (39/81 samples). The bacterium was found in 76% of environmental water samples (16/21) and was especially common in waste water (80%). It was also found in 35% (23/60) of drinking water samples, according to the criteria adopted. Sixty-two percent (24/39) of *H. pylori*-positive samples were positive for *cagA*. Only 1% of samples was positive for *H. pylori* by culture, 47% by nested PCR, 41% by real-time PCR, 43% by RT-nested PCR, 37% by RT-real-time PCR and 31% by IFA. There was a high prevalence of virulent *H. pylori* in the water samples in this region, implying that drinking water and environmental water may be important sources for this pathogen, potentially leading to gastroduodenal or hepatobiliary diseases.

Keywords: *Helicobacter pylori*, environment, drinking water, nested PCR, real-time PCR

1. INTRODUCTION

Helicobacter spp. can colonize the leading to several diseases including gastric gastrointestinal and hepatobiliary tracts, cancer and cholangiocarcinoma (CCA) [1, 2].

Helicobacter pylori is the main causative agent of gastroduodenal diseases and hepatobiliary diseases [2]. The global prevalence of *H. pylori* infection is more than 50% and is highest in underdeveloped and developing regions [3, 4]. Transmission is widely assumed to be via the oral-oral or fecal-oral routes [3]. Some epidemiological studies have shown that *H. pylori* infection is associated with the level of sanitation, particularly water sanitation, especially in developing countries [5, 6].

H. pylori is a fastidious microorganism and requires a nutrient-rich medium and a microaerobic environment for growth [7]. It can enter a viable but not culturable (VBNC) state, during which it will not actively grow on conventional media but remains metabolically active. This leads to difficulty in culturing and isolation from environmental samples [3]. Molecular-based approaches, such as conventional PCR and real-time PCR, are widely used as alternative tools for detection of *H. pylori* and for identification of strains harboring important virulence-associated genes such as the cytotoxin-associated gene A (*cagA*) [8].

Molecular detection of *H. pylori*, generally targets the vacuolating cytotoxin gene A (*vacA*) [9] and the 16s rRNA gene [10]. Hence, in this study, *vacA* (nested PCR) and 16s rRNA (SYBR green real-time PCR) genes were targeted to detect the presence of *H. pylori* in water samples. Viability of *H. pylori* in water samples can be assessed using reverse-transcription PCR (RT-PCR), [11], and indirect fluorescent antibody (IFA) assays [12].

The IFA test uses specific antibodies for detection of bacteria in environmental samples [13]. This method allows the enumeration of both culturable and nonculturable cells and both the spiral and coccoid forms of *H. pylori* [12], but cannot detect virulence marker genes such as *cagA*.

A high prevalence of virulent *H. pylori* isolates has been reported from gastric cancer and hepatobiliary patients and from asymptomatic Thai individuals in northeastern Thailand [14, 15]. This prompted our investigation of the environmental reservoir of the bacteria to clarify the sources of infection. There has been no previous report of *H. pylori* in environmental water and drinking water in Thailand. The aims of this study were to investigate the prevalence of *H. pylori* carrying a virulence-associated gene, *cagA*, in the water samples using PCR-based methods (nested PCR, real-time PCR, RT-nested PCR and RT-real-time PCR) as well as the IFA assay, and to compare the prevalence of *H. pylori* in different classes of water sources.

2. MATERIALS AND METHODS

2.1 Bacterial Strains

The bacterial strains used in this study were obtained from American type culture collection (ATCC), Department of Medical Sciences Thailand (DMST) Culture Collection, and clinical and environmental sources at Srinagarind Hospital and Department of Microbiology, Faculty of Medicine, Khon Kaen University, Thailand. The human ethics committee at our institution reviewed and approved the present study (HE 561037).

2.2 Sample Collection and Processing

Eighty-one samples from environmental sources and drinking water were aseptically collected in or near the municipality of Khon Kaen, northeastern Thailand, between September 2013 and February 2014 and transported to the laboratory within 2 hours at 4 °C. Environmental samples ($n = 21$) came from 13 ponds, 3 canals and 5 waste-water reservoirs. Drinking water samples ($n = 60$) were from 25 food shops/food vendors, 17 water coolers in houses, 5 factories and

13 drinking water vending machines. Sites for water sampling had to satisfy one or more of the following criteria; 1) a previous cholera outbreak had been linked to the site, 2) non-epidemic area, 3) nearby high population density, such as on or near a university campus or villages, and 4) the site was a community drinking water factory. The sampling location was randomly selected in a 50 kilometer radius around Muang District, Khon Kaen Province.

In brief, each 500 ml water sample was filtered serially through Whatman filter paper No.1, 0.45 μm cellulose acetate membrane filter (Whatman, UK), and 0.2 μm pore-size filters (Sartorius, Germany). The membranes were placed in 25 ml sterile Brucella broth and incubated at 37 °C under aerophilic and microaerophilic conditions for 0, 3 and 7 days for enrichment. The enriched culture was then subdivided as follows; (i) 1 ml for further culture, (ii) 2 ml for DNA extraction, (iii) 2 ml for RNA extraction and (iv) 2 ml for the IFA assay.

2.3 Culture Media

Culture media including HP medium [16], modified Columbia urea agar (MCUA) [17], blood agar and Brucella agar were slightly modified by adding growth supplements for *H. pylori* (strains DMST 20165, DMST 20979) and changing the indicator reagents (Table 1).

2.4 Oligonucleotide Primers and Specificity

Oligonucleotide primers specific for the genus *Helicobacter* (16S rRNA) and for *H. pylori* (16S rRNA and *vacA*) and the PCR conditions used are shown in Table 2. All primers were commercially synthesized by 1st BASE, Singapore. The specificities of primers were checked using the *in silico* BLAST program on the NCBI database and an *in vitro* PCR assay employing 100 ng of DNA template

of three *H. pylori* strains (DMST 20165, DMST 20979 and a clinical isolate), and 20 other bacterial strains [18].

Table 1. Modified plating media and enrichment media for selection and recovery of *H. pylori* from mixed bacterial populations in spiked water samples.

| | Additional reagent in conventional media (per liter) | | | | | | |
|--|--|------------------------|----------------------|---|--------------------|---------------------|--------------------------------------|
| | Indicator: 2,3,5-Triphenyltetrazolium chloride (40 mg) | Sodium pyruvate (10 g) | L-cysteine (10 mmol) | Ammonium Sulfate ($(\text{NH}_4)_2\text{SO}_4$) (10 mmol) | Vancomycin (10 mg) | Trimethoprim (5 mg) | Amphotericin B Polymixin B (3,500 U) |
| Conventional culture media | + | + | - | - | + | + | + |
| Modified HP medium ^a | + | + | + | - | + | + | + |
| Modified Columbia urea agar ^b | + | + | + | - | + | + | + |
| Blood agar | + | + | + | - | + | + | + |
| Brucella agar | + | + | + | - | + | + | + |
| Brucella broth | - | - | - | + | + | + | + |

^a Final pH 6.0 at 22°C [16]; ^b Prepared according to [17] with slight modification.

Table 2. Primer sequences and PCR conditions for amplification of *Helicobacter* spp. and *H. pylori* used in this study.

| Target organisms | Genes | Primer sequences (5'→ 3') | Amplicon sizes (bp) | PCR condition | References |
|--------------------------|--|-----------------------------|---------------------|------------------------------------|------------|
| <i>Helicobacter</i> spp. | 16S rRNA (Nested PCR) | OF-ATTAGTGGCGCACGGGTGAGTAA | 1,300 | 94°C 30 s, 55°C 30 s, | [2] |
| | | OR-TTTAGCATCCCGACTTAAGGC | | 72°C 1.5 min (35 cycle) | |
| | | IF-GAACCTTACCTAGGCTTGACATTG | 480 | 94°C 30 s, 60°C 30 s, | |
| | | IR-GGTGAGTACAAGACCCGGGAA | | 72°C 30 s (35 cycle) | |
| <i>H. pylori</i> | Universals <i>vacA</i> (Nested PCR) | OF-GCATGATTTTGGCACCATTG | 429 | 94°C 15 s, 60°C 20 s, | [18] |
| | | OR-TTTTCATATTTAGGGGCAAA | | 72°C 30 s (35 cycle) | |
| | | IF-GCATGATTTTGGCACCATTG | 276 | 94°C 15 s, 60°C 20 s, | |
| | | IR- ATCGCATTGCTCAAGCTCAA | | 72°C 30 s (35 cycle) | |
| <i>H. pylori</i> | 16s rRNA (real-time PCR) | F-GAAGATAATGACGGTATCTAAC | 139 | 95°C 5 min; 95°C 15 s, | [19] |
| | | R-ATTTTCACACCTGACTGACTAT | | 58°C 20 s, 72°C 30 s (40 cycle) | |
| <i>H. pylori</i> | Universals <i>cagA</i> (Nested PCR) | OF- AGACAACCTGAGCGAGAAAG | 1233 | 94°C 30 sec, 57°C 30 sec, | [18] |
| | | OR- TGTTGAGCCAATTGCTCCTTT | | 72°C 30 sec (35 cycle) | |
| | | IF- AGACAACCTGAGCGAGAAAG | 307 | 94°C 15 s, 57°C 20 s, | [2] |
| | | IR- GGAGGCGTTGGTGTATTGA | | 72°C 30 s (35 cycle) | |

2.5 Sensitivity of RT-nested/ RT-SYBR Green qPCR from Pure Culture

The detection limits of reverse transcription (RT)-nested PCR (*vacA*) and RT-real-time PCR (16s rRNA gene) assays were assessed using serial dilutions of pure culture (10^6 - 10^0 CFU/ml) in sterile phosphate-buffered saline (PBS). RNA template was extracted from each diluted sample using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The RNA pellet was dissolved in RNase-free water before treatment with DNase using RQ1 RNase-free DNase (Promega, Madison, WI). The mRNA was converted to cDNA using superscript III reverse transcriptase (Life Technologies, Grand Island, NY) according to the manufacturer's instructions. The generated cDNA was used as template for assessment of sensitivity of the RT-nested PCR and RT-real-time PCR assays.

2.6 PCR Assays

Nested PCR for amplification of 16s rRNA for *Helicobacter* spp., universal *vacA* and *cagA* genes was conducted in a 15 µl reaction mixture containing 0.2 mM of dNTPs, 1× PCR buffer, 1.5 mM MgCl₂, 0.2 µM primers (16s rRNA of *Helicobacter* spp. and *vacA* assays) or 0.5 µM primers (*cagA* assay), 0.5 units of *Taq* polymerase (RBC Bioscience, Taipei, Taiwan) and 100 ng of genomic DNA extracted using a commercial DNA extraction kit (Pure Gene DNA purification kit, Qiagen, USA) or 200 ng of cDNA template. Purified genomic DNA or cDNA of *H. pylori* reference strains or sterile distilled water was included in each set of reactions as positive and no-template controls, respectively. Thermocycling (Bio-Rad C1000, Hercules, CA) was performed as shown in Table 2. PCR products were separated by 1.5% agarose gel electrophoresis, stained with ethidium bromide and visualized under

UV transilluminator.

The RT- and/or real-time PCR targeting the 16s rRNA of *H. pylori* was performed as previously described [19]. In brief, SYBR-green real-time PCR was conducted in a total volume of 15 μ l comprising 7.5 μ l of 2X Maxima™ SYBR Green/ROX qPCR Master Mix (Fermentas Life Science, Canada), 0.5 μ M primers and 100 ng of genomic DNA or 200 ng of cDNA template. Nuclease-free water was used as the no-template control. Thermocycling (ABI 7500 thermocycler Applied Biosystems) was performed as shown in Table 2. The melting curve analysis of the target amplicon was 86.5 ± 0.5 °C.

2.7 *Helicobacter pylori* Isolation and Identification from Water Samples

A 1-ml aliquot of enrichment culture in Brucella broth was centrifuged at $13,000 \times g$ for 2 min, then the pellet was resuspended in 250 μ l PBS. Numbers of *H. pylori* cells in the PBS suspension was determined by the dilution plate count and streak-plate technique on modified HP selective medium after incubation at 37 °C for 3 - 7 days under microaerobic conditions (5% CO₂; 10% H₂; 85% N₂) [4, 16]. The suspected gold metallic-looking colonies and other pink colonies were collected for further identification using biochemical tests such as catalase, oxidase, and urease production [8], and for DNA extraction.

2.8 Indirect Fluorescence Antibody (IFA) for Detecting *H. pylori*

The IFA test for the detection of *H. pylori* was performed according to the previously described procedures with slight modifications [13]. Briefly, 1 ml of *H. pylori* culture was added with 12.5 μ l of 0.025% yeast extract (Difco, USA) and 10 μ l of 0.002% nalidixic acid (Sigma, USA). Following

overnight incubation at 25 °C in dark conditions, each sample was fixed using formaldehyde followed by methanol and then incubated with 1:100 mouse monoclonal IgG anti-*H. pylori* antibody (Santa Cruz, USA) and 1:200 Goat anti-mouse IgG1-conjugated FITC for 1 h at 4 °C and 25 °C, respectively, in a dark and moist chamber. Each slide was then washed and air dried, mounted with fluorescent mounting solution (New Horizons Diagnostics Corporation, USA) and observed under a fluorescence microscope (Nikon). The specificity of IFA was determined with the same bacterial strains used for evaluation of specificity of the PCR assay. Cells of *H. pylori* were identified on the basis of three criteria; (i) intense bright green fluorescence, (ii) size (1 - 3 μ M) and (iii) shape (curved-rod shape or coccoid forms) [20].

To detect *H. pylori* in environmental and drinking water samples by IFA, an aliquot (2 ml) of each water sample was centrifuged at $13,000 \times g$ for 2 min. Then the pellet was suspended in 500 μ l of PBS solution prior to mixing with 6.25 μ l of 0.025% yeast extract and 5 μ l of 0.002% nalidixic acid. A 30- μ l aliquot of mixture was then treated and examined as described previously.

2.9 Sequence Analysis

Amplicons of 16s rRNA, *vacA* and *cagA* generated by nested PCR were confirmed by sequencing analysis (First BASE, Malaysia). Multiple alignment of DNA sequences and of translated protein sequences was done using ClustalW within BioEdit Sequence Alignment Editor version 7.0.5.3 and Molecular Evolutionary Genetic Analysis (MEGA) version 4 software [21]. For comparing and analyzing the nucleotide sequences, the BLAST program at the NCBI website was employed.

3. RESULTS AND DISCUSSION

In this study, the modified HP medium (as shown in Table 1) adapted from the conventional medium was optimized [16] for recovery and selection of *H. pylori* from water samples. This medium can detect the pathogen at a concentration of 10^3 - 10^4 CFU/ml, whereas typical colonies were not seen using other media (modified Columbia urea agar, Blood agar, Brucella agar) (Table 1). Plates positive for *H. pylori* (DMST strain 20165) showed dark red colonies with a golden shine (Figure 1A), whereas an *H. pylori* reference strain recovered from a spiked water sample showed dark red colonies without a golden shine (Figure 1B). Several reagents were added into the modified HP medium, such as whole blood or serum, to support growth of the bacterium [22], antibiotics as selective

agents for *H. pylori* [7], sodium pyruvate to enhance growth and aerotolerance by neutralizing the toxic effect of oxygen [7], L-cysteine to stimulate the recovery of *H. pylori* and decrease coccoid forms [22], and 2,3,5-triphenyltetrazolium chloride as an indicator to identify *H. pylori* as colonies with a golden shine [4]. Despite the development of this supplemented medium, only one water sample (a drinking water sample) yielded a culture of *H. pylori*. The low rate of culture success might be due to 1) bacterial factors including cells entering the viable but non-culturable state, cells dying, or viable cell density being below the detection limit of the culture method, or 2) the properties of the culture medium itself rendering it unsuitable for growth of *H. pylori* in real water samples.

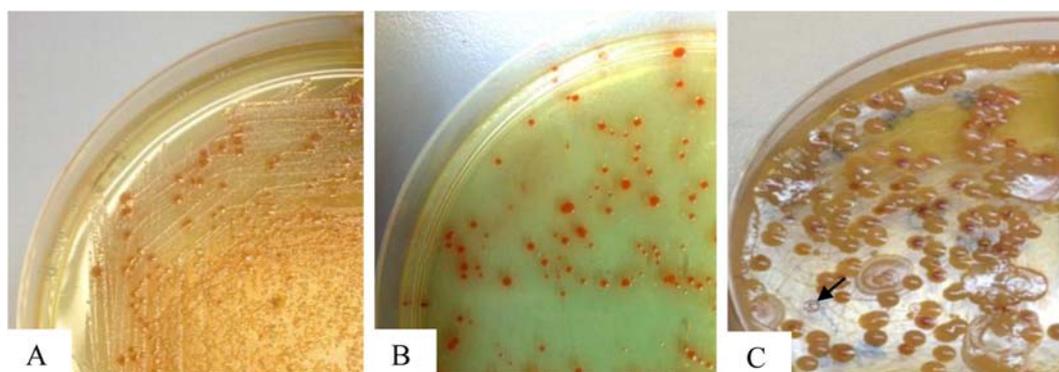


Figure 1. Growth of *H. pylori* on modified HP agar plates containing 2, 3, 5-triphenyltetrazolium chloride indicator. 1A, *H. pylori*-positive plate (strain DMST 20165 from pure culture) showing dark red colonies with a golden shine; 1B, example of *H. pylori* reference strain recovered from a spiked water sample showing dark red colonies without golden shine; 1C, *H. pylori* isolated from drinking water showing pink colonies (indicated by arrow).

This reflects findings of other studies showing that *H. pylori* is rarely isolated by culture from environmental water samples. For example, *H. pylori* was cultured from only 2.8% and 1.2% of environmental water samples using HP medium and modified Columbia agar, respectively [8] and from

2.5% of samples of drinking water from water coolers, taps and dental unit water supplies in Iran using Brucella agar [6].

It is of interest that *H. pylori* colonies from spiked environmental samples had a distinct morphology and produced urease enzyme at a very low level (> 12 h). Colony

morphology might have been influenced by environmental factors [23] and the expression of urease activity might be affected by growth conditions [4].

Previous study has shown that detection of *vacA* and 16s rRNA improved sensitivity and specificity of recognition of *H. pylori* in samples containing many other microorganisms as well as many organic impurities [19]. The *vacA* is a major virulence gene found in all known *H. pylori* strains and serves as a species-specific gene [4]. The 16s rRNA gene is universal in bacteria, but differs somewhat from species to species. Therefore, this target gene could be used to identify *H. pylori* when directly amplified from water samples [8]. In this study, four PCR primer sets, specific at the genus level for *Helicobacter*, at the species level for *H. pylori* (nested PCR and real-time PCR) and specific for *cagA* were tested against three reference *H. pylori* strains and 20 different bacterial species. No product band was detected in any species tested other than *H. pylori* (data not shown).

The limit of detection of the RT-nested PCR (*vacA*) was 10^4 CFU/ml (6×10^2 CFU/PCR) and of the RT-SYBR green qPCR assay (16s rRNA gene) was 10 CFU/ml (~ 1 CFU/PCR). There have been no previous reports on the detection limits of these RT-PCR based methods for *H. pylori* from pure culture [19, 24], therefore, comparisons cannot be made. However, previous study showed that the lowest number of cells from a pure culture detectable by nested PCR was 14 cells [25] and for real-time PCR was 10^3 cells [26].

In this study, *H. pylori* was detected in 39 out of 81 water samples (48%) collected from environmental sources and drinking water according to the criteria chosen (a positive result in IFA and at least one of the

molecular methods, or according to both molecular methods). Of 39 *H. pylori*-positive samples, 36 (92%) contained viable cells of the bacterium when assayed using culture, RT-nested PCR, RT-SYBR green qPCR and IFA. The overall prevalence of viable *H. pylori* was therefore 44% (36/81). For each method, the result is shown in Table 3. This prevalence is higher than previously detected in drinking water (23%) from Peru using RT-PCR [11].

Relative sensitivities of the nested PCR, real-time PCR and IFA were differed. Significant differences ($P < 0.05$) were noted in the number of positive samples according to real-time PCR and IFA compared with prevalence of viable *H. pylori* as well as RT-real-time PCR and IFA compared with the number of true positive (TP) using the McNemar test (Table 3). Average numbers of viable *H. pylori* cells from various water sources detected using RT-SYBR green qPCR were between 10^2 and 10^3 CFU/ml (Table 4). Previous reports showed that the minimum infective dose was 10^4 cells in specific-pathogen *H. pylori* in monkeys [27] and was more than $10^3 - 10^4$ CFU/ml from *H. pylori*-infected persons [28].

The IFA assay can detect bacteria in both viable and VBNC forms and can be used to observe the coccoid and rod shapes of *H. pylori* from colonies and directly from water samples [12]. RT-nested PCR, RT-real time PCR and IFA were employed to detect viable cells whereas the conventional nested PCR and conventional real-time PCR can be used for the detection of both viable and dead organisms. Therefore, two or more methods should be combined to enhance the specificity of *H. pylori* detection in water samples, and to increase confidence in the results.

Table 3. Comparisons of methods for detection of *H. pylori* in 81 water samples.

| Culture | Methods | | | Total Number (%) | Evaluation |
|---------|----------------------------|--------------------------|---------------------|-----------------------|------------|
| | Nested PCR (<i>vacA</i>) | Real-time PCR (16s rRNA) | IFA | | |
| + | + (1) ^a | + (1) ^b | + | 1 (1) [□] | TP |
| | + (15) ^a | + (14) ^b | + | 16 (20) [□] | TP |
| | + (12) ^a | + (13) ^b | - | 15 (19) [□] | TP |
| | + | - | + | 3 (4) | TP |
| | - | + (2) ^b | + | 4 (5) [□] | TP |
| | - | - | + | 1 (1) | FP |
| | + | - | - | 3 (4) | FP |
| | - | + | - | 3 (4) | FP |
| | - | - | - | 35 (43) | TN |
| 1 (1%) | 34 (42%) ^c | 30 (37%) ^{c**} | 24 | 36 (44%) ^e | 39 (48%)* |
| | 37 (46%) ^d | 33 (41%) ^{d**} | (30%) ^{**} | | |

TP, True positive; TN, True negative; FP, False positive; *, number and percentage of TP samples. Note: TP = positive by IFA and at least one of the molecular methods, or by both molecular methods; [□], True positive regarded as containing viable *H. pylori*

^a, positive by nested PCR and reverse-transcription (RT)-nested PCR

^b, positive by real-time PCR and RT-real-time PCR

^c, positive by RT-nested PCR and RT-real-time PCR

^d, positive by nested PCR and real-time PCR

^e, prevalence of viable *H. pylori*

^{**}, Significant differences ($P < 0.05$) in detection rate compared with prevalence of viable *H. pylori* (^c compared with ^e) and number of TP* (^d compared with ^{*}) using the McNemar test.

Table 4. Quantification of viable *H. pylori* in various types of water sample, Northeast Thailand ^a.

| Water source | No. of positive samples | Average number of viable <i>H. pylori</i> by RT-real-time PCR (CFU/ml) |
|----------------|-------------------------|--|
| Pond | 6 | 2.3×10^3 |
| Canal | 2 | 6.9×10^2 |
| Waste | 3 | 4.8×10^2 |
| Drinking water | 13 | 1.7×10^3 |

^a 24 of 30 positive water samples were analyzed.

The overall prevalences of *Helicobacter* spp. and *H. pylori* were 53% (43/81) and 48% (39/81), respectively. Among the 81 samples tested, *Helicobacter* spp. were found in 90% (19/21) of environmental water samples and 40% (24/60) of drinking water samples, whereas *H. pylori* was found in 76% (16/21) of environmental water samples and in 35% (23/60) of drinking water samples (Table 5). Of 39 *H. pylori*-positive samples, 62% (24/39) carried *cagA*: over half (57%) of *H. pylori*-positive drinking

water samples were also positive for this gene (Table 5). Our results therefore indicate that the drinking waters in this region harbor virulent *H. pylori* carrying *cagA*, a gene associated with gastroduodenal

and hepatobiliary disease [4, 14]. This is in agreement with a study in Iran, which found *cagA*-carrying *H. pylori* in bottled mineral water at the high rate of 62.5% [29].

Table 5. Distribution of *Helicobacter* spp. and *H. pylori* in environmental and drinking water samples.

| Water source | No. of samples | No. of <i>Helicobacter</i> spp. positive (%) | No. of <i>H. pylori</i> positive (%) | No. of <i>cagA</i> -positive <i>H. pylori</i> (%) |
|----------------|----------------|--|--------------------------------------|---|
| Environmental | 21 | 19 (90) | 16 (76) | 11 (69) |
| Pond | 13 | 12 (92) | 10 (77) | 8 (80) |
| Canal | 3 | 2 (67) | 2 (67) | 2 (100) |
| Waste | 5 | 5 (100) | 4 (80) | 1 (25) |
| Drinking water | 60 | 24 (40) | 23 (35) | 13 (57) |
| Total | 81 | 43 (53) ^a | 39 (48) ^a | 24 (62) ^b |

^a, compared to all water sources

^b, 24 of 39 (62%) *H. pylori* were *cagA*-positive.

In Peru, *H. pylori* was found in 50% of drinking water samples by PCR [11] whereas in Iran, *H. pylori* was found in 4%, 6% and 3% of tap water, dental units' water, and bottled mineral water samples [6], respectively. The presence of *H. pylori* in water samples thus varies geographically. This may be dependent on the environmental conditions in each region, such as physiochemical factors, pH, temperature, type of water, and also the methods and testing processes used including the sensitivity and specificity of primers.

Helicobacter spp. in patients or in the water have been reported to have an association with the severity and prevalence of diseases especially in *cagA*-positive *H. pylori* [4, 20]. The high prevalence of *Helicobacter* spp. in drinking water warrants further study on identity and pathogenicity because species such as *H. bilis* and *H. hepaticus* can be found in bile and hepatobiliary tissues of patients with biliary tract diseases including biliary tract cancer [30].

The *vacA* and *cagA* genes were sequenced from all positive samples and compared with reference nucleotide sequences on the NCBI database. The *vacA*-positive water samples showed 95 - 97% identity with *vacA* of reference *H. pylori* strains: *cagA* sequences exhibited 96 - 99% identity with *cagA* of *H. pylori* F32 reference strain (accession no. AP011943.1). The *cagA* sequence of the Thai *H. pylori* environmental isolate showed differences at positions 1362 (A→G), 1374 (G→A), 1375 (G→A) and 1515 (T→C), leading to an amino acid change at position 459 (Valine → Isoleucine). This change might affect the virulence of the bacterium and pathogenesis in the host.

The presence of *H. pylori* in water samples correlates with the prevalence of this species in gastrointestinal tract and hepatobiliary tract patients in Northeast Thailand [31, 32]. *H. pylori* infection was detected in 44.3% of gastric biopsy samples [31], a value similar to that reported between

2008 and 2013 in four regions of Thailand (45.9%) [32]. Infection with *H. pylori* may be related to poor sanitary practices [5]. The highest level of *H. pylori* infection was associated with lowest household income and failure to boil drinking water [33]. Therefore, the distribution of *H. pylori* in environmental and drinking waters should be monitored and the sanitary practices of people should be improved to decrease morbidity rate of *H. pylori* infection.

4. CONCLUSIONS

In conclusion, to the best of our knowledge, this is the first report of the prevalence of *H. pylori* from water samples in Thailand. PCR-based assays are very efficient for detecting *H. pylori* in aquatic environments compared to IFA and culture. Many drinking water samples contained *H. pylori* carrying the virulence gene *cagA*. These findings indicate that drinking water and aquatic environments may be reservoirs of pathogenic *H. pylori* that could transmit to humans living in conditions of poor sanitation. Thus, the presence of *H. pylori* in water samples may be related to gastroduodenal and hepatobiliary diseases. Surveillance of *H. pylori* in the environment and drinking water should be ongoing.

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