RESEARCH ARTICLE

High Prevalence of *Helicobacter pylori* Resistance to Clarithromycin: a Hospital-Based Cross-Sectional Study in Nakhon Ratchasima Province, Northeast of Thailand

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Abstract

Background: *Helicobacter pylori* is a cause of chronic gastritis, peptic ulcer disease, and gastric malignancy, infection being a serious health problem in Thailand. Recently, clarithromycin resistant *H. pylori* strains represent the main cause of treatment failure. Therefore this study aimed to determine the prevalence and pattern of *H. pylori* resistance to clarithromycin in Suranaree University of Technology Hospital, Suranree University of Technology, Nakhon Ratchasima, Northeastern Thailand, Nakhon Ratchasima province, northeast of Thailand. Materials and Methods: This hospital-based cross-sectional study was carried out between June 2014 and February 2015 with 300 infected patients interviewed and from whom gastric mucosa specimens were collected and proven positive by histology. The gastric mucosa specimens were tested for *H. pylori* and clarithromycin resistance by 23S ribosomal RNA point mutations analysis using real-time polymerase chain reactions. Correlation of eradication rates with patterns of mutation were analyzed by chi-square test. Results: Of 300 infected patients, the majority were aged between 47-61 years (31.6%), female (52.3%), with monthly income between 10,000-15,000 Baht (57%), and had a history of alcohol drinking (59.3%). Patient symptoms were abdominal pain (48.6%), followed by iron deficiency anemia (35.3%). Papaya salad consumption (40.3%) was a possible risk factor for *H. pylori* infection. The prevalence of *H. pylori* strains resistant to clarithromycin was 76.2%. Among clarithromycin-resistant strains tested, all were due to the A2144G point mutation in the 23S rRNA gene. Among mutations group, wild type genotype, mutant strain mixed wild type and mutant genotype were 23.8%, 35.7% and 40.5% respectively. With the clarithromycin-based triple therapy regimen, the efficacy decreased by 70% for *H. pylori* eradication (P<0.01). Conclusions: Recent results indicate a high rate of *H. pylori* resistance to clarithromycin. Mixed of wild type and mutant genotype is the most common mutant genotype in Nakhon Ratchasima province, therefore the use of clarithromycin-based triple therapy an not advisable as an empiric first-line regimen for *H. pylori* eradication in northeast region of Thailand.

Keywords: Prevalence - *Helicobacter pylori* - clarithromycin resistance - hospital-based cross-sectional study - Thailand

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Introduction

Helicobacter pylori is a gram-negative, helical shaped and microaerophilic bacterium that colonizes in the pathogenesis of chronic gastritis, peptic ulcer disease, and gastric malignancy (Komoto et al., 1998). Since the discovery of *Helicobacter pylori* in 1983, strong evidence has indicated that *H. pylori* eradication is current standard treatment and can prevent of chronic gastritis, peptic ulcer recurrence and malignancy change (Mihara et al., 1999). Prevalence of *H. pylori* infection has geographic variation in which there is a higher prevalence in the developing country including Thailand (Tan and Goh, 2008). The distribution pattern of *H. pylori* infection ranges from 25 to 50% in developed countries to more than 80% in the developing world (Beswick et al., 2006). Although the bacterium is susceptible to most antimicrobial agents *in vitro*, but the successful treatment of *H. pylori* is a challenge (Gerrits et al., 2006). *H. pylori* treatment has been regimented that clarithromycin common practicum, however, clarithromycin resistant *H. pylori* strains represent the main cause of treatment failure and eradication. The primary resistance rate of *H. pylori* to clarithromycin is different among each region of the world. The overall global resistance from a systemic review in 2004 was 9.9% (95% CI 8.3-11.7) (Megraud, 2004), and

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during 2006-2009 was 17.2% (95% CI 16.5-17.9) (De Francesco et al., 2010). A highest of resistance rate was found in Japan, while, the lowest rate was found in Korea and Thailand (Kobayashi et al., 2002). Nationwide survey of *H. pylori* antibiotic resistance in Thailand was reported. The result show that antibiotic resistance was presented in 50.3% including metronidazole 36%, ciprofloxacin 7.7%, levofloxacin 7.2%, amoxicillin 5.2%, multi-drugs in 4.2%, clarithromycin 3.7%, and tetracycline 1.7%, respectively (Vilaichone et al., 2013). Therefore, this study aimed to determine the prevalence of *H. pylori* to clarithromycin resistance associated with 23S ribosomal RNA point mutations, and the effect on clarithromycin base triple therapy for *H. pylori* eradication in Nakhon Ratchasima province, northeast region of Thailand.

Materials and Methods

Population and Diagnosis of Helicobacter pylori associated gastritis

A hospital-based study was carried out among 300 patients diagnosed with *H. pylori* during June 2014 and January 2015. The study was performed in Suranaree University of Technology hospital, Suranree University of Technology, Nakhon Ratchasima, Northeastern Thailand. All patients provided a written informed consent. A diagnosis of *H. pylori* associated gastritis was made if *H. pylori* were seen on histopathological examination and the rapid urease test were positive. Finally, we prove bacterial infection by Polymerase chain reaction (PCR) method. Briefly,

Biopsy specimens and histological analysis

Biopsy was done according to the updated Sydney classification system (Dixon et al., 1994), which indicates sampling from 5 biopsy sites, each specimen should be obtained from the lesser curvature of the corpus about 4 cm proximal to the angulus (1), from the lesser curvature (2) and greater curvature of the antrum (3), both within 2 to 3 cm of the pylorus, from the middle portion of the greater curvature of the corpus, approximately 8 cm from the cardia (4), and from the incisura angularis (5). Gastric tissue specimens for histological analysis were sent to pathologist. The hematoxylin and eosin stain and Giemsa stain were used for identification of *H. pylori*. The pathological analysis made by 5 pathologist of Bangkok Pathological Laboratory outside Suranaree University of technology.

DNA isolation method Real Time PCR methods

The DNA of *H. pylori* was extracted from gastric biopsy specimen using the QIAamp DNA FFPE tissue kit (Qiagen, USA). The DNA extraction was performed according to manufacturer protocol. Briefly, ten tissue sections of 5 μM thick were collected in 1.5 ml microcentrifuge tubes. The deparaffinization step was accomplished by xylene and 95% ethanol. The protein digestion step was done by incubation the tissue in proteinase K digestion buffer at 56°C and 90° C for 1 hour of each step. Then the tissue solution was transferred to spin QIAamp MinElute column to let the DNA combine to the membrane. The samples were then subjected to centrifugation at 6000 × g. After that the samples were subjected to repeated washing step by using the provided washing buffers. In the final step was DNA samples were eluted using elution buffer at the final volume of 50 μl. The detection of 23S rRNA mutation was performed, by using the real-time PCR technique. The hybridization fluorescent probe was utilized for PCR product detection. The real-time PCR procedure was accomplished by using Light Cycler® 480 instrument (Roche diagnostics, Neuilly sur Seine, France). The identifications of target PCR products were accomplished by melting curve analyses. The target PCR products were amplified by using the primers HPYS and HPYA as previously reported in the previous literature (Me nard et al., 2002). PCR-RFLP can also detect the point mutation A2142C of the 23S rRNA gene associated with resistance of *H. pylori* to clarithromycin. The amplified products have a size of 267 bp. The hybridization probes include the one that is in the mutation sites of the 23S rRNA gene of *H. pylori*, the sensor probe. The sequence is 5-GGCAAGACGGAAAGACC-3; nucleotides 2504 to 2520). This sensor probe is labeled by LC-red 640 at 5' and phosphorylated at 3'. The anchor probe will hybridized to the PCR product at the site 3 bp upstream to the sensor probe. The probe sequence is 5 TGTAGTGGAGGTGAAAATTCCTCCTACCC-3; nucleotides 2473 to 2501, GenBank accession number U27270). The probe is labeled with fluorescein at 3'. 3 μl DNA templates were subjected to PCR reaction in the final volume of 20 μl. The reaction mixture consists of MgCl2 (25 mM), forward and reverse primers (20 M each), sensor and anchor probes (20 M each), and 2 μl of FastStart DNA Master Hybridization Probes (Roche Diagnostics). PCR amplification comprised an initial denaturation cycle at 95°C for 10 min, followed by 50 amplification cycles (with a temperature transition rate of 20°C/s) consisting of 95°C for 0 s, annealing at 60°C for 10 s, and extension at 72°C for 17 s. After amplification a melting step was performed, consisting of 95°C for 0 s, cooling to 45°C for 30 s (with a temperature transition rate of 20°C/s), and finally a slow rise in the temperature to 85°C at a rate of 0.1°C/s with continuous acquisition of fluorescence decline. According to previous report using this real-time PCR protocol, this melting curve analysis can detect the possible three mutant geneotypes along with the wild type according to different Tm. The reported Tm of the wild type, A2121C, A2142G and A2143G were 61.5, 58.0, 53, 53.6 °C respectively.

Statistical analysis

SPSS for Windows (version 16.0; SPSS, Chicago, IL, USA) was used for the statistical analysis; baseline demographic data of the infected patients were analyzed. The correlation of eradication rate and pattern of mutation were analyzed by chi-square test. All results were considered statistically significant when the P-values were less than 0.05.

Results

A total of 300 *H. pylori* associated gastritis patients

enrolled in this study. The most of patients was female and 45.2 years old. The majority of patient was age between 47-61 years old (31.6%), and followed by 62-76 years old (25.3%). Female (52.3%) was slightly higher than male (47.6%). The most of them had a monthly income between 10,000 - 10,000 bath (57%), and 10,000 - 15,000 bath (33.6%). The majority of them had a history with alcoholic drinking (59.3%), and followed by smoking (32.6%), spicy food (4.6%). Patients' symptom was presented abdominal pain (48.6%), and followed by iron deficiency anemia (35.3%), and vomiting (8%), respectively. The most of them was identified a non-ulcer gastritis/duodinitis (84%), and followed by peptic ulcer disease (GU/DU). They had a mean follow-up time 33±4 day. Papaya salad consumption (40.3%) was the majority of a possible risk factor, and followed by pickled fish (34%), food street vendor (9.3%), respectively (Table 1). Among clarithromycin-resistant strains tested, all were due to A2144G point mutation in 23S rRNA gene. The prevalence of *H. pylori* strains resistant to clarithromycin was 76.2%. The most of them was wild type + mutation (susceptible + resistance) (40.5%), followed by mutation, A2143/2142CG (resistance) (35.7%), and wild type (23.8%), respectively (Table 2). The pattern of clarithromycin resistance by using real-time PCR hybridization probe method and correlation with *H. pylori* eradication rate was show in Table 2, Figure 1, and 2 respectively. Clarithromycin-based triple therapy regimen, the efficacy decreased by 70% for *H. pylori* eradication (P<0.01).

Table 1. Demographic data of Helicobacter pylori infected patients in Suranaree University of Technology Hospital, Nakhon Ratchasima province, Northeast of Thailand during June 2014 and February 2015

Patient's demographic data	Helicobacter pylori infected patients n (%) Total number: 300
Age	
17-31 year	65 (21.6%)
32-46 year	64 (21.3%)
47-61 year	95 (31.6%)
62-76 year	76 (25.3%)
Sex	
Male	143 (47.6%)
Female	157 (52.3%)
Income	
<5,000 bath/month	10 (3.3%)
5,000-10,000 bath/month	171 (57%)
10,000-15,000 bath/month	101 (33.6%)
>15,000 bath/month	18 (6%)
Personal history	
Smoking	98 (32.6%)
Alcoholic drinking	178 (59.3%)
High temperature food intake	10 (3.3%)
Spicy food	14(4.6%)
Symptom	
Abdominal pain	146 (48.6%)
Iron deficiency anemia	106 (35.3%)
Gastrointestinal bleeding	24 (7.9%)
Vomiting	16 (8%)
Diarrhea	8 (2.6%)
Possible risk factor	
Family history	4 (1.3%)
Food street vendor consumption	28 (9.3%)
Farmer	16 (5.3%)
Pickled fish consumption	102 (34%)
Salt crab consumption	16 (5.3%)
Papaya salad consumption	121 (40.3%)
Thai vermicelli eaten with curry	9 (3%)
Vegetarian food	4 (1.3%)
Identification	
Peptic ulcer disease (GU/DU)	45 (15%)
Gastric cancer	3 (1%)
Non ulcer gastritis/duodinitis	252 (84%)

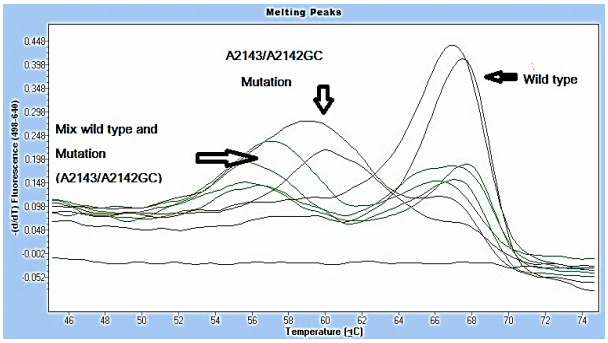

Figure 1. Pattern of Clarithromycin Resistance by Using Real-time PCR Hybridization Probe Method

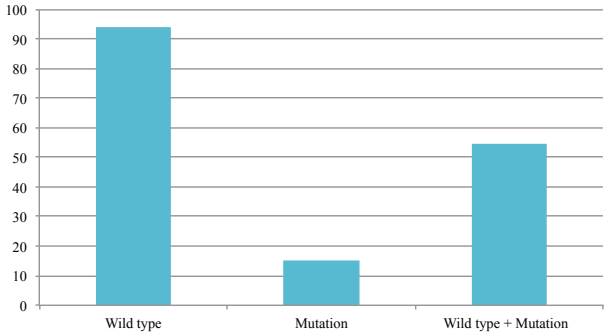

Figure 2. Correlation between Mutation type and Helicobacter Pylori Eradication Rate

Table 2. Mutation pattern and Helicobacter pylori eradication rate in the infected patients in Suranaree University of Technology Hospital, Nakhon Ratchasima province, Northeast of Thailand during June 2014 and February 2015

Test susceptible/resistant to Clarithromycin	n = 300	Helicobacter pylori eradication rate
Wild type, A2143/2142A (Susceptible)	23.80%	93.80%
Mutation, A2143/2142CG (Resistance)	35.70%	15.30%
Wild type + Mutation (Susceptible + Resistance)	40.50%	54.70%

Discussion

H. pylori is remains a major public health problem in Thailand. Recently, the results show that 84% and 15% of them were the non-ulcer gastritis/duodinitis, and peptic ulcer disease. Unfortunately, 1% of them was the gastric cancer, this figure indicates that *H. pylori* infection was a serious problem in the northeastern Thailand where has the high incidence of cholangiocarcinoma; bile ducat cancer. In addition, the new discovery has been a strong, positive correlation between opisthorchiasis-associated cholangiocarcinoma and infection with Helicobacter. The findings indicate that the liver fluke Opisthorchis viverrini in the biliary tree of the hamsters harbors *H. pylori* and Helicobacter-like bacteria. Accordingly, the association between O. viverrini and *H. pylori* may be an obligatory mutualism (Deenonpoe et al., 2015). Recent results showed the majority of them had a history with alcoholic drinking, smoking, and spicy food consumption. Previous studied had shown that smoking has effects in *H. pylori* related gastric carcinogenesis. The smoking behavior contributed to the increased risk of gastric carcinogenesis from gastric atrophy, and had little influence on *H. pylori* infection or gastric atrophy development (Hishida et al., 2010).

Alcohol consumption has a close relationship with peptic ulcer diseases. Chronic active gastritis is reportedly associated with chronic alcohol ingestion. Nonetheless, the inflammatory changes are likely to be related to concurrent *H. pylori* infection that is common among alcoholics. Moreover, chronic alcoholism is also correlated with the presence of gastric metaplasia. Both clinically and experimentally, alcohol had been shown to affect the mucosal barrier and histology. These ulcerogenic effects play a crucial role in altering gastric mucosal defense mechanisms. Concurrent consumption of alcohol and cigarette smoking significantly increases the risk of gastric ulcers. In animal experiments, cigarette smoking potentiated ethanol-induced gastric mucosal damage. The reduction of mucus secretion, increase in leukotriene B4 level, increased activities of inducible nitric oxide synthase, xanthine oxidase and myeloperoxidase, and the expression of adhesion molecules in the gastric mucosa accompanied such potentiating effects (Ko and Cho, 2000).

Among clarithromycin-resistant strains tested, all were due to A2144G point mutation in 23S rRNA gene. The most of them was wild type + mutation (susceptible + resistance), followed by mutation, A2143/2142CG (resistance), and wild type. This result is similar to Wang et al (2000), *H. pylori* strain resistant to metronidazole and clarithromycin in Hong Kong. Dual resistant strains reduced the eradication rate to 66.7%. Among clarithromycin-resistant strains tested, all were due to A2144G point mutation in 23S rRNA gene. A meta-analysis of 93 studies with 10,178 subjects has been reported (Fischbach et al., 2007). Clarithromycin resistance affects the efficacy of first line triple therapy regimen as well. In standard, clarithromycin base triple therapy regimen, the efficacy decreased by an estimated 66 % (95% CI 58.2-74.2) and the eradication rate ranged 0%-50%. A standard clarithromycin base triple therapy recommended by American College of Gastroenterology guideline (Chey et al., 2007) and the second Asia-Pacific consensus for *H. pylori* eradication (Fock et al., 2009), however, antibiotic-resistant *H. pylori* strain increasing, the efficacy of this regimen can be reduced. Nationwide survey of antibiotic resistance to *H. pylori* in Thailand has been reported and found that the antibiotic resistance was 50% and these bacteria resistant to 3.7% clarithromycin (Vilaichone et al., 2013). Recent result, the prevalence of *H. pylori* strains resistant to clarithromycin was 76.2%. The clarithromycin-based triple therapy regimen, the efficacy decreased by 70% for *H. pylori* eradication. According to this result, the majority of histologically-proven *H. pylori* infected cases (76.2%) have mutant genotypes, which confer the resistant property to clarithromycin, the clinical data indicated that most of the cases poor response to the treatment for standard regimen. This observation indicated that even in the cases who have resistant strain, this treatment protocol (clarithromycin base triple therapy) an ineffective to eradicate the bacteria in our area and answer clinical practice outcome. The underlying reasons could be explained these hypotheses, the possible reason that underlies the mixed geneotypes are multiple infections of the same patient by two strains. The other is the occurring of mutation after one infection. The further genotypic analyses have to pursue to confirm these possible mechanisms.

In conclusion, prevalence of clarithromycin resistant *H. pylori* is geographically different among regions of Thailand. Physician should be concern of local resistance prevalence in each area and choose the most appropriate regimen for *H. pylori* eradication. Larger multi-center studies about mutation pattern are needed to test this hypothesis.

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