RESEARCH ARTICLE

Effect of Pretreatment with *Lactobacillus delbrueckii* and *Streptococcus thermophillus* on Tailored Triple Therapy for *Helicobacter pylori* Eradication: A Prospective Randomized Controlled Clinical Trial

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Abstract

Background: *Helicobacter pylori* plays an important role in gastric cancer and typical eradication regimens are no longer effective in many countries, including Thailand. The aim of our study was to compare the effect of *Lactobacillus delbrueckii* and *Streptococcus thermophillus* on tailored triple therapy for *Helicobacter pylori* eradication. Materials and Methods: This prospective single-center study was conducted in Thailand. *Helicobacter pylori* associated gastritis patients were randomized to 2 groups: group 1 (n=100) was tailored triple therapy with placebo (esomeprazole 20 mg bid, clarithromycin 500 mg bid or metronidazole 400 mg tid if clarithromycin resistance and amoxicillin 1000 mg bid), and group 2 was tailored triple therapy plus pretreatment with probiotic containing yogurt. Successful eradication was defined as both negative histology and negative rapid urease test at four weeks after treatment. Results: A total of 200 infected patients were enrolled. PP analysis involved 194 patients: 96 in the tailored triple therapy with placebo group (group 1) and 98 the in tailored triple therapy plus pretreatment with probiotic containing yogurt group (group 2). Successful eradication was observed in 170 (87.6%) patients; by PP analysis, the eradication rate was significantly higher in group 2 (P = 0.04, 95%CI; 0.02-0.13) than in group 1. ITT analysis also showed that the value was significantly higher in the tailored triple threapy plus pretreatment with probiotic containing yogurt group (group 2) (89/100; 89%) than in the tailored triple therapy with placebo group (group 1) (P= 0.01, 95%CI; 0.04-0.15). In terms of adverse events, there was no significant difference between the two groups. Conclusions: Pretreatment with probiotic containing yogurt can improve *Helicobacter pylori* eradication rates with tailored triple therapy. Adding probiotics does not reduce adverse effects of the medication.

Keywords: Pretreatment with probiotics - *Helicobacter pylori* - tailored triple therapy - gastric cancer

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Introduction

Since the discovery of *Helicobacter pylori* in 1983, strong evidence has indicated that *Helicobacter pylori* infection plays an important role in the pathogenesis of chronic gastritis and gastric malignancy (Komoto et al., 1998). It currently infects more than half of the world's population, and in the last decade it has been recognized as a major human pathogen (Mihara et al., 1999). *Helicobacter pylori* eradication is currently the standard treatment and can prevent chronic gastritis, peptic ulcer recurrence and malignant change (Mihara et al., 1999). *Helicobacter pylori* has proven difficult to cure and standard triple therapy is no longer recommended as an empiric choice in most countries (Chey et al., 2007). The effectiveness of the most commonly used therapies has been increasingly compromised by the rapid emergence of antibiotic resistant strains of *Helicobacter pylori* and by poor compliance with treatment by patients (Mégraud et al., 2013). However, triple therapy is still recommended in areas where clarithromycin resistance is low, or when therapy is chosen based on pretreatment susceptibility testing. Resistance to amoxicillin has remained relatively stable, while resistance rates to clarithromycin have been steadily increasing (De Francesco et al., 2007; Lee et al., 2013; Su et al., 2013; Megraud et al., 2013). Many reports

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suggest that probiotics compete directly with *Helicobacter pylori* by interfering with *Helicobacter pylori* adherence or by producing antimicrobial molecules. A recent meta-analysis investigated whether a preparation of a Lactobacillus and Bifidobacterium containing probiotic could improve *Helicobacter pylori* eradication rates and reduce adverse events (Wang et al., 2013). They concluded that combining a Lactobacillus and Bifidobacterium containing probiotic with initial *Helicobacter pylori* eradication therapy in adults may have beneficial effects on the eradication rate and the incidence of total adverse events. Clarithromycin resistance against *Helicobacter pylori* is associated with point mutations in the 23S ribosomal RNA (rRNA) gene. When a point mutation occurs, the binding of clarithromycin to the ribosome decreases causing resistance (Occhialini et al., 1997). Prescribing an antibiotic for *Helicobacter pylori* eradication based on susceptibility testing is an approach that has been used clinically, allowing "tailored treatment" with marked improvements in treatment success. Indeed, high eradication rates have been obtained by tailoring the triple therapy to the resistance patterns of *Helicobacter pylori* (Kato et al., 2004; Claudia Schabereiter-Gurtner et al., 2004). A study performed in Thailand showed that the 7-day standard triple therapy plus bismuth and a probiotic can provide excellent cure rates for *H. pylori* (100%) since the country has low clarithromycin resistance rates (Srinarong et al., 2014). However, the clarithromycin resistance rates vary in Thailand based on geography, especially in our area.

This is the first study to evaluate whether the addition of probiotic containing yogurt, (Suranaree brand) made by the Suranaree Farm, Suranaree University of Technology, Nakhornhachasima, Thailand which contains Lactobacillus delbrueckii subp. bullgaricus and Streptococcus thermophillus, to tailored triple therapy beneficially affects *Helicobacter pylori* eradication rates.

Materials and Methods

Patients

A total of 200 patients diagnosed with *Helicobacter pylori* associated gastritis participated in this study from June 2014 through January 2015. The following exclusion criteria were applied: age below 18 or above 70 years, previous *Helicobacter pylori* eradication treatment, gastric ulcer or duodenal ulcer, suspected or confirmed malignancy on endoscopy, significant medical illnesses history of previous gastric surgery, pregnant or lactating women, the use of antimicrobials or gastrointestinal medications like PPIs or bismuth compounds within the previous 2 months, refusal of yogurt due to underlying diseases such as DM, or history of drug allergy to one of the first line therapies. The study was performed in accordance with good clinical practice and the guidelines of the Declaration of Helsinki. All patients provided written informed consent and the study protocol was approved by the Ethics Committee for Research Involving Human Subjects, Suranaree University of Technology (EC-57-22) and the Thai Clinical Trials Registry (number TCTR20141211001).

Diagnosis of Helicobacter pylori associated gastritis

A diagnosis of *Helicobacter pylori* associated gastritis was made if *Helicobacter pylori* bacteria were seen on histopathological examination and the rapid urease test was positive. A recent study from India (Patel SK et al., 2014) attemoted to define the "gold standard" of diagnostic tests to determine *Helicobacter pylori* infection status depending on the sensitivity and specificity. Both sensitivity and specificity of nested PCR has been reported to be 100%. In contrast, the sensitivity and specificity of serological, urea breath, fecal antigen, rapid urease tests, histopathology, PCR and culture have been found to be 85% and 79%, 75%-100% and 77%-100%,67%-100% and 61%-100%, 75%-100% and 84%-100%,66%-100% and 94%-100%, 75%-100% and 84%-100% and 55%-56% and 100%, respectively. The PCR seems to not be feasible in daily clinical practice due to cost and availability. In our study, patients with negative results in one or both examinations were considered to be *Helicobacter pylori* negative.

Biopsy specimens

Biopsy was done according to the updated Sydney classification system (Dixon et al.,1996) which indicates sampling from 5 biopsy sites: one specimen each 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)

Histological analysis

Gastric tissue specimens for histological analysis were sent to the pathologist. The hematoxylin and eosin stain and Giemsa stain were used for identification of *Helicobacter pylori*. The pathological analysis was made by 5 pathologists at Bangkok Pathological Laboratory outside of Suranaree University of Technology.

DNA isolation method

The DNA of *Helicobacter pylori* was extracted from frozen gastric tissue biopsy specimens which were stored at a temperature of less than -20°C using the QIAamp DNA FFPE tissue kit (Qiagen, USA). The DNA extraction was performed according to the manufacturer protocol. Briefly, ten tissue sections of 5 μM thick were collected in 1.5 ml micro centrifuge tubes. The tissue specimens were placed in a microcentrifuge tube, and buffer ATL (180 μL) and proteinase K (20 μL) were added. The samples were mixed by vortexing and incubated at 56°C until the tissues were completely lysed. Buffer AL (200 μL) was added to the samples, which were subsequently incubated at 70°C for 10 minutes. Next, 240 μL of 100% ethanol was added to the samples, which were mixed by vortexing for 15 seconds. Each sample was placed in a QIAamp spin column and centrifuged at 8000 rpm for 1 minute. The columns were washed with AW1 buffer (500 μL), and samples were centrifuged at 8000 rpm for 1 minute. AW2 buffer (500 μL) was added to the column, and samples were centrifuged at 14 000 rpm for 3 minutes. Buffer AE

(200 μL) was added to each sample, and samples were incubated for 1 minute prior to centrifugation at 8000 rpm for 1 minute. Finally, the DNA was extracted from the tissue.

Detection of point mutations in the 23S rRNA gene of Helicobacter pylori by real-time PCR

The mutation detection of 23S Rena gene was performed by using the real-time PCR technique for template amplification. The hybridization fluorescent probe was utilized for PCR product detection. The real-time PCR procedure was accomplished by using a LightCycler® 480 instrument (Roche diagnostics, Neuilly sur Seine, France). The identification of target PCR products was 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. 27PCR-RFLP can also detect the point mutation A2142C of the 23S rRNA gene associated with resistance of *Helicobacter pylori* to clarithromycin. The amplified products have a size of 267 bp.The hybridization probes include the one that is at 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 a previous report using this real-time PCR protocol, this melting curve analysis can detect all three of the possible mutant genotypes along with the wild type according to different Tm. The reported Tm of the wild types, A2121C, A2142G and A2143G were 61.5, 58.0, 53, 53.6 °C respectively.

Probiotic containing yogurt

The yogurt contains Lactobacillus delbrueckii subp. bullgaricus and Streptococcus thermophillus with an inoculation rate of 50u/250ml. Lactobacillus delbrueckii subp. bullgaricus (> 10 5CFU/serve) and Streptococcus thermophillus (> 10 8CFU/serve) were obtained from the Suranaree Farm, Suranaree University of Technology, Nakhornhachasima, Thailand. It lasts at least 24 months from the date of manufacture when stored according to recommendations. At +50C (410F) the shelf life is at least 6 weeks.

Symptoms and safety evaluation

The study was performed in accordance with good clinical practice and the guidelines of the Declaration of Helsinki. All patients provided written informed consent and the study protocol was approved by the Ethics Committee for Research Involving Human Subjects, Suranaree University Of Technology (EC-57-22). All patients were asked to report associated symptoms at baseline and during follow-up, including diarrhea, metallic taste, nausea/vomiting and rash. Any adverse events related to therapy were recorded and analyzed.

Study design

This randomized, prospective, single center study was conducted at the Endoscopic unit, Suranaree University of Technology Hospital (SUTH) located at Suranaree University of Technology, Nakhonrachasima province in Thailand. 200 *Helicobacter pylori* associated gastritis patients were randomized into two groups using the Random Number Generator by SPSS for Windows (version 16.0; SPSS, Chicago, IL, USA): group 1 was given one week of placebo (yogurt without probiotic) followed by one week of tailored triple therapy (esomeprazole 20 mg bid, clarithromycin 500 mg bid or metronidazole 400 mg tid if clarithromycin resistance and amoxicillin 1000 mg bid; group1, n=100), group 2 was given one week of pretreatment with probiotic containing Lactobacillus delbrueckii subp. bullgaricus and Streptococcus thermophillus followed by one week of tailored triple therapy as above (group2, n=100) (Figure1). After completion of the therapeutic protocol, rapid urease test and biopsy were performed by gastroscopy at least 4 weeks later because we wanted to evaluation the gastric mucosal morphologic pattern after treatment with probiotic to answer the second objective of this study. Diagnosis of *Helicobacter pylori* associated gastritis was positive if seen on the histopathological examination and the rapid urease test was also positive. Patients with negative results in one or both examinations were considered to be *Helicobacter pylori* negative. At the time of enrollment, a personal interview was conducted and a questionnaire was administrated. Patients were informed of the importance of full compliance, warned of adverse events, instructed to complete treatment, and provided with a contact number, in case they encountered a problem. One week after completion of the tailored triple therapy, compliance and adverse events for two groups were evaluated by direct questioning by a physician and pill counting. Patients were

Table 1. Patient Baseline Demographics (PP, per-protocol-analysis)

Patient baseline demographics data	Tailored triple therapy with placebo (n=96)	Tailored triple therapy with probiotic(n=98)	P-value
Male/female (n)	39/57	42/56	0.14
Mean age (years)	45.2	47.5	0.22
Mean follow-up time,(day)	33±4	35±2	0.28

Table 2. Pattern of Clarithromycin Resistance

Test susceptible/resistant to Clarithromycin	Tailored triple therapy with placebo (n=96)	Tailored triple therapy with probiotic(n=98)	P-value
Wild type , A2143/2142A(Susceptible)	11	12	0.32
Mutation, A2143/2142CG(Resistance)	25	24	0.32
Wild type + Mutation (Susceptible + Resistance)	60	62	0.15

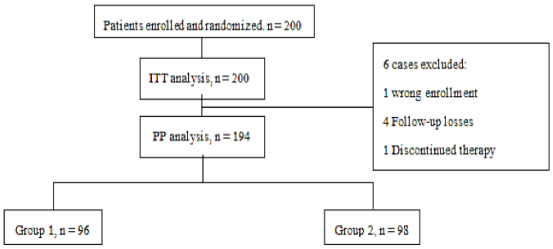

Figure 1. Flow Diagram Showing Numbers of patients enrolled and missed for Procol and Intention-to-treat Analyses. ITT: Intention-to-treat; PP: Per-protocol. Group1: Tailored triple Therapy alone, Group 2 Tailored triple theraply plus yogust

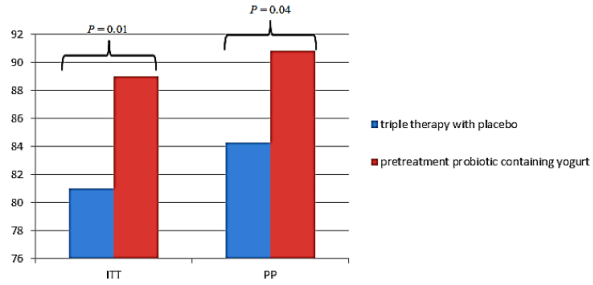

Figure 2. Helicobacter Pylori Eradication Rate (ITT, Intention-to-treat; PP: Per-protocol)

Table 3. Adverse Events of Patients in Two Groups

Adverse event	Tailored triple therapy with placebo (n=96)	Tailored triple therapy with probiotic(n=98)
Diarrhea	25%	26%
Metallic taste	64%	61%
Nausea /Vomiting	28%	24%
Rash	4%	6%

asked for the details of gastrointestinal symptoms and aggravated baseline symptoms that developed during the first week. Compliance was considered to be satisfactory when drug and yogurt intake exceeded 90%.

Statistical analysis

The eradication rates of *Helicobacter pylori* were determined by ITT and PP methods. All enrolled patients were included in the ITT analysis. However, for the PP analysis, patients that were lost to follow up, had taken less than 90% of the prescribed drugs or yogurt, or those that had dropped out due to adverse events were excluded. SPSS for Windows (version 16.0; SPSS, Chicago, IL, USA) was used for the statistical analysis. The eradication rate, baseline demographic data of the ITT and PP populations were compared by Student's t tests. The eradication rate and 95% confidence intervals in each group were calculated for both the PP and ITT populations. All results were considered statistically significant when the P-values were less than 0.05.

Results

Patient population

Figure 1 shows a schematic diagram of this study. A total of 200 *Helicobacter pylori* associated gastritis patients enrolled into the study. Among these patients, 100 were assigned to one week with placebo before tailored triple therapy (group1), 100 patients to one week pretreatment with probiotic containing yogurt before the tailored triple therapy (group2). The demographic data of the 2 study groups are summarized in Table 1 and patterns of Clarithromycin resistance are summarized in Table 2. Sex, the mean age of the patients, mean follow-up time and clarithromycin resistance of the two groups were similar.

Helicobacter pylori eradication

Four weeks after the completion of tailored triple therapy, by PP analysis *Helicobacter pylori* testing by rapid urease test and biopsy were negative in 170 (87.62%) of the 194 patients. The results showed that the eradication rates were significantly higher in the pretreatment with probiotic containing yogurt group (group2) (89/98, 90.8%) than in the pretreatment with placebo group (group1) (81/96, 84.3%) (P=0.04, 95%CI 0.02-0.13)(Figure 2). ITT analyses showed that, compared with the tailored triple therapy with placebo group (81/100, 81%) success rates were significantly higher in the pretreatment with probiotic containing yogurt group (group2) (89/100, 89%) (P=0.01, 95%CI 0.04-0.15)

Symptoms and safety assessment

The percentage of patients with adverse events in each group is shown in Table 3. There were no significant differences between the treatment and placebo groups.

Discussion

It has been 30 years since the discovery of *Helicobacter pylori* in 1983 by Australian physicians Robert Warren and Berry Marshal (Marshall BJ et al., 1984). The International Agency of Cancer classified *Helicobacter pylori* as a Class I carcinogen for gastric cancer in 1994 (Schistosomes et al., 1994). Since then the bacteria has been thought to be one of the causative factors in the development of gastric cancer. Infection with *Helicobacter pylori* causes chronic inflammation and significantly increases the risk of developing duodenal and gastric ulcer disease and gastric cancer. *Helicobacter pylori* infection is the strongest known risk factor for gastric cancer, which is the second leading cause of cancer-related deaths worldwide (Wroblewski LE et al., 2010). *Helicobacter*

pylori eradication can reduce the risk of gastric cancer. In clinical practice, *Helicobacter pylori* infection has proven difficult to cure due to drug resistance.

The benefit of probiotics for *Helicobacter pylori* eradication was first discovered following a series of research studies in germ-free mice. The studies reported that *Helicobacter pylori* colonizes germ-free but not SPF mice, and that Lactobacillus in the stomach of SPF mice inhibits colonization by *Helicobacter pylori*. Pre-treatment with probiotics has been found to significantly reduce the *Helicobacter pylori* colonization rate in mice, from 100% to 50% (P=0.02) and reduce inflammation grading in the gastric antrum (Johnson-Henry KC et al.2004). Recent evidence revealed that the administration of probiotics increases eradication rates by anti *Helicobacter pylori* therapy. Tong et al. (2007) A meta-analysis of supplemental probiotics in eradication therapy including 14 randomized trials showed that eradication rates for triple therapy alone and eradication therapy plus probiotics were 74.8 and 83.6%, respectively. With combined treatment, the eradication rate increased, and adverse effects, such as diarrhea decreased. However, the eradication rate varies by protocol. Sheu et al (Sheu B et al., 2006) reported that pretreatment with Lactobacillius and Bifidobacterium-containing yogurt improved the efficacy of quadruple therapy after failed triple therapy. They also demonstrated a decreased bacterial load after pretreatment with yogurt.

In many countries including Thailand, standard triple therapy with a clarithromycin-containing regimen is associated with an eradication rate less than 80% according to many factors especially clarithromycin resistant bacteria.26 Therefore, we chose a protocol involving pretreatment with Lactobacillus delbrueckii subp. bullgaricus and Streptococcus thermophillus containing yogurt in group 2 when compared with tailored triple therapy with placebo (group1). ITT analysis also showed that eradication rates were significantly higher in the pretreatment with probiotic group (group 2) than in the tailored triple therapy with placebo group (group1) (P=0.01), by PP analysis, the eradication rates were also significantly higher in group 2 (P=0.04) than in the placebo group (group1). However in term of adverse events, the result showed that there was no difference between the two groups.

According to our results using real-time PCR, the majority of histologically-proven *Helicobacter pylori* infected cases have mutant genotypes, which confer resistance to clarithromycin. However, the clinical data indicates that most of the cases still respond well to the treatment protocol which replaced clarithromycin with another antibiotic accompanied by probiotic treatment. This observation indicates that even in cases which have a resistant strain, this treatment protocol is still effective in eradicating the bacteria. The underlying reasons could be explained by these hypotheses. First, the clarithromycin resistant strains may still be sensitive to the alternate antibiotic regimen included in the treatment protocol so the patients can be effectively treated. The second possibility is that the infection process can be influenced by adding the probiotics in the treatment protocol. The effect of each mechanism per se or the combination of both can also play a role. The possible reason that underlies the mixed genotypes is multiple infections of the same patient by two strains. The other is the occurring of a mutation after the infection. A further genotypic analysis is needed to pursue and confirm these possible mechanisms.

In conclusion, our data suggested that pretreatment with probiotic containing Lactobacillus delbrueckii subp. bullgaricus can improve the eradication rate of *Helicobacter pylori* and associated gastritis in patients both by PP and ITT analysis, however there was no significant difference between the two groups in terms of adverse events. Probiotic Lactobacillus delbrueckii subp. bullgaricus and Streptococcus thermophillus containing yogurt (Suranaree brand) is effective as an addition to treatment with tailored triple therapy for *Helicobacter pylori* associated gastritis patients.

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