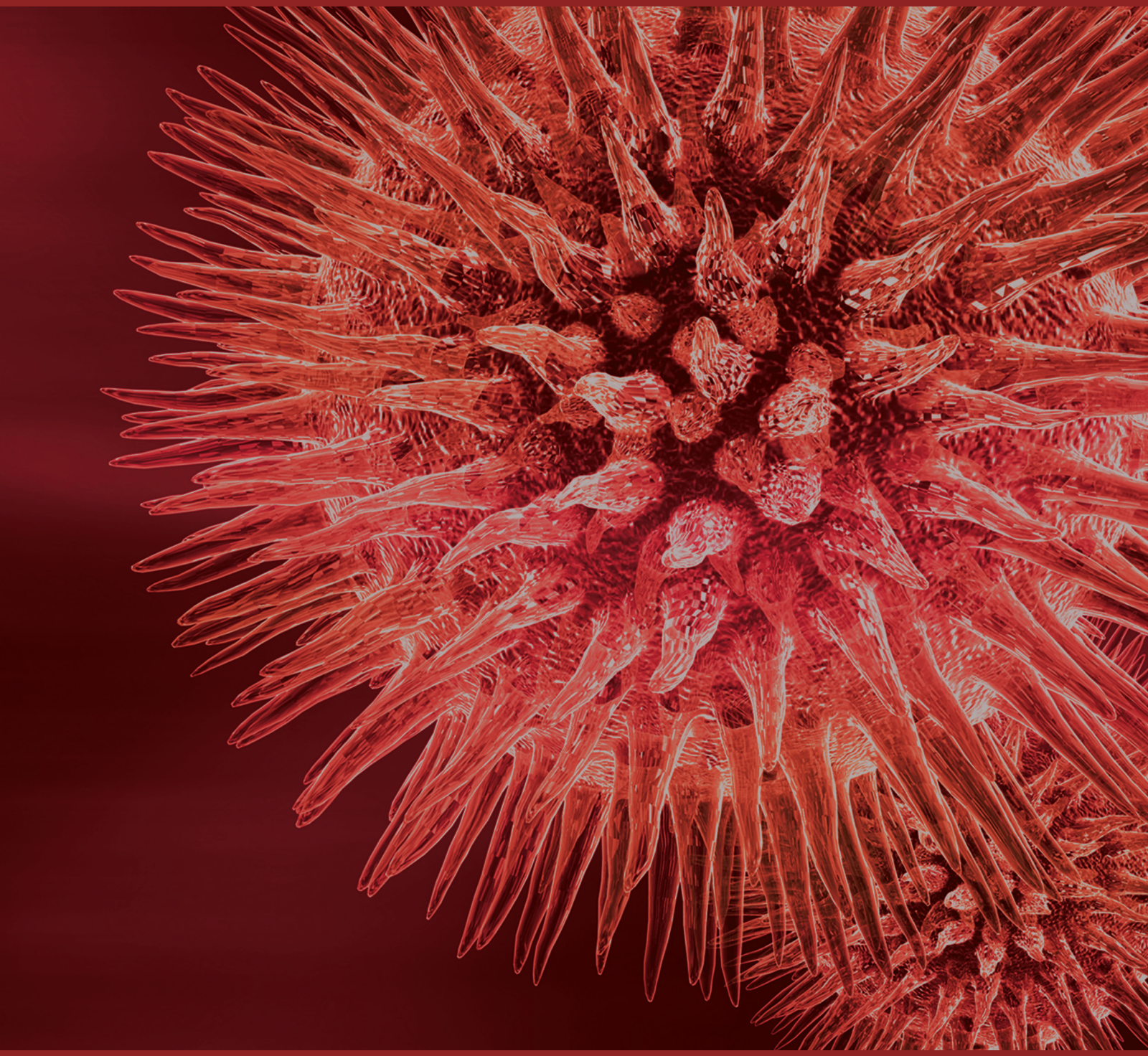
BioMed Research International

Helicobacter pylori Infection

Guest Editors: Ping-I Hsu, Yoshio Yamaoka, Khean-Lee Goh, Marco Manfredi, Deng-Chyang Wu, and Varocha Mahachai



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Editorial
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Helicobacter pylori (*H. pylori*) colonizes the gastric mucosa of more than 50% of the human population. It is the major etiological agent of chronic gastritis, peptic ulcer, gastric mucosa-associated lymphoid tissue lymphoma, and gastric adenocarcinoma. The concomitance of particular genotypes of both pathogen and host may lead to the development of serious gastroduodenal diseases.

With the rising prevalence of antimicrobial resistance, the treatment success of standard triple therapy has recently declined to unacceptable levels in most countries. Several strategies including sequential, concomitant, and hybrid therapies are therefore proposed to increase the eradication rate of first-line treatment for *H. pylori* infection. Since the best first-line eradication regimen with the highest eradication rate and low adverse effects remains unclear and the exact route of transmission is still not exactly known, *H. pylori* infection continues to be a big challenge to all gastroenterologists 30 years after its discovery.

The main focus of the special issue is on recent advances in the diagnosis and treatment of *H. pylori* infection. In addition, the virulence factors and transmission of *H. pylori* are also discussed.

T. Tongtawee et al. in "Correlation between Gastric Mucosal Morphologic Patterns and Histopathological Severity of *Helicobacter pylori* Associated Gastritis Using Conventional Narrow Band Imaging Gastroscopy" investigated specific gastric mucosal morphologic patterns of *Helicobacter pylori* gastritis by NBI. The data indicate that mucosal morphologic patterns of *H. pylori* gastritis can be reliably identified using C-NBI gastroscopy with good correlation with inflammation grading.

W.-C. Tai et al. in "Seven-Day Nonbismuth Containing Quadruple Therapy Could Achieve a Grade "A" Success Rate for First-Line *Helicobacter pylori* Eradication" conducted a prospective trial to compare the efficacies of nonbismuth containing quadruple therapy and standard triple therapy in Taiwan. The results showed that a 7-day nonbismuth containing quadruple therapy achieved a higher eradication rate than 7-day standard triple therapy (95.6% versus 79.3% by per-protocol analysis). In the paper entitled "Levofloxacin-Amoxicillin/Clavulanate-Rabeprazole versusa Standard Seven-Day Triple Therapy for Eradication of *Helicobacter pylori* Infection," M.-C. Chen et al. demonstrated that a seven-day regimen containing levofloxacin, amoxicillin/clavulanate, and rabeprazole was superior to a standard triple regimen containing clarithromycin, amoxicillin, and rabeprazole in Taiwan.

Regarding the second-line therapy, G.-H. Jheng et al. in "Comparison of Second-Line Quadruple Therapies with or without Bismuth for *Helicobacter pylori* Infection" conducted a randomized controlled trial to compare the efficacies of standard quadruple regimen (rabeprazole, bismuth

subcitrate, tetracycline, and metronidazole) and a modified concomitant regimen (rabeprazole, amoxicillin, tetracycline, and metronidazole) after failure of standard triple therapy. Intention-to-treat analysis showed that the two rescue quadruple therapies had comparable eradication rates (91.9% and 89.7%, resp.). The results suggest that the 10-day modified concomitant regimen can be an alternative rescue therapy for *H. pylori* infection in bismuth-unavailable countries. In the paper entitled "Quinolone-Containing Therapies in the Eradication of *Helicobacter pylori,*" S.-K. Chuah et al. review the efficacies of quinolone-containing regimens for *H. pylori* infection and discuss the public health issue of emerging resistant strains of mycobacteria following quinolone-containing *H. pylori* eradication therapy.

In the paper entitled "The Prevalence of *Helicobacter pylori* Virulence Factors in Bhutan, Vietnam, and Myanmar Is Related to Gastric Cancer Incidence," T. T. H. Trang et al. examined the status of *cagA*, *vacA*, *jhp0562,* and *β-(1,3)galT* in *H. pylori*-infected patients from Bhutan, Vietnam, and Myanmar. The data suggest that the *cagA*, *vacA* s1, *vacA* m1, and *jhp0562*-positive/*β-(1,3)galT*-negative may play a role in the development of gastric cancer.

Biofilm formation is critical not only for environmental survival but also for successful infection. H. Yonezawa et al. in "Biofilm Formation by *Helicobacter pylori* and Its Involvement for Antibiotic Resistance" demonstrated that biofilm formation of *H. pylori* could decrease susceptibility to antibiotics and *H. Pylori* antibiotic resistance mutations were more frequently generated in biofilms than in planktonic cells.

In the paper entitled "Helicobacteraceaein Bulk Tank Milk of Dairy Herds from Northern Italy," V. Bianchini et al. revealed that *H. pylori* was not identified in any of the samples from the bulk tank milk of dairy cattle herds. The data suggest that, at least in the farming conditions of the investigated area, bovine milk does not represent a potential source of infection.

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Review Article

Biofilm Formation by *Helicobacter pylori* and Its Involvement for Antibiotic Resistance

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Bacterial biofilms are communities of microorganisms attached to a surface. Biofilm formation is critical not only for environmental survival but also for successful infection. *Helicobacter pylori* is one of the most common causes of bacterial infection in humans. Some studies demonstrated that this microorganism has biofilm forming ability in the environment and on human gastric mucosa epithelium as well as on *in vitro* abiotic surfaces. In the environment, *H. pylori* could be embedded in drinking water biofilms through water distribution system in developed and developing countries so that the drinking water may serve as a reservoir for *H. pylori* infection. In the human stomach, *H. pylori* forms biofilms on the surface of gastric mucosa, suggesting one possible explanation for eradication therapy failure. Finally, based on the results of *in vitro* analyses, *H. pylori* biofilm formation can decrease susceptibility to antibiotics and *H. pylori* antibiotic resistance mutations are more frequently generated in biofilms than in planktonic cells. These observations indicated that *H. pylori* biofilm formation may play an important role in preventing and controlling *H. pylori* infections. Therefore, investigation of *H. pylori* biofilm formation could be effective in elucidating the detailed mechanisms of infection and colonization by this microorganism.

1. Introduction

Helicobacter pylori is a spiral, microaerophilic, noninvasive, gram-negative bacterium that colonizes the human gastrointestinal tract, primarily the stomach [1]. *H. pylori* is one of the most common causes of human infection, especially in developing countries, where the incidence can be up to 90% of the population [2]. *H. pylori* infection often persists throughout life. This organism has been identified as an etiological agent of chronic active gastritis, peptic ulcer disease [3, 4], gastric adenocarcinoma [5], and mucosa-associated lymphoid tissue (MALT) lymphoma [6]. In addition, a working group of the World Health Organization International Agency for Research on Cancer concluded in 1994 that *H. pylori* is a group I definite carcinogen in humans [7]. Even though most individuals infected with *H. pylori* are asymptomatic, infected individuals form a high-risk population for the above-mentioned diseases. A number of factors such as the vacuolating cytotoxin, the *cagA* and *cag* pathogenicity island (*cag*PAI), motility, adhesins, and the urease enzyme are known to be involved in the virulence of this organism [8]. *H. pylori* exists in two morphological forms [9]. One is a spiral form and the other is a nonculturable but viable coccoid form. The spiral form is the most common form involved in colonization of the human stomach. It has been reported that, for survival under unsuitable conditions, this microorganism has the ability to convert its spiral form to the coccoid form [9–13].

Recently, some studies have alluded to the ability of *H. pylori* to form biofilms *in vitro* [14–16]. In addition, *H. pylori* can form biofilms on the human gastric mucosa [17–19]. Moreover, *H. pylori* could be embedded in drinking water biofilms on the surfaces of water distribution systems in developed and developing countries [20]. Therefore, a more thorough understanding of *H. pylori* biofilm should provide useful information for the characterization of this microorganism. In this review, several scientific observations including our research data on *H. pylori* biofilm formation will be described. In addition, a novel eradication strategy for *H. pylori* biofilm will be suggested.

2. Bacterial Biofilm Formation

Most bacteria live under severe nutrient-limited conditions. To protect themselves from hostile environmental influences, bacteria often form surface attached communities described as "bacterial biofilms." Biofilms are ubiquitous in natural, industrial, and clinical environments and have been shown to play a critical role in many chronic infections [21]. Biofilms are usually composed of multiple bacterial species. For example, dental biofilms (i.e., dental plaque) contain more than 500 different bacterial species [22]. Biofilms consist of viable microbial cells along with dead cells and a wide range of self-generated extracellular polymeric substances (EPS) including polysaccharides, nucleic acids (extracellular DNA from bacteria), and proteins [23]. The EPS matrix can constitute up to 90% of the biofilm biomass. The initial attachment is driven by hydrophobic or electrostatic interactions as well as specific bacterial surface molecules. The next step is multiplication of the bacteria and formation of microcolonies with EPS surrounding the microcolonies. In the third step (maturation step), the biofilm forms thick and mushroom-like or tower-like structures with increasing numbers of bacteria. Subsequently, the enlarged biofilm shows focal dissolution and liberates planktonic bacterial cells which can spread to other locations.

Biofilm bacteria exhibit distinct properties which differ from those of planktonic cells [24, 25]. One of these is an increased resistance to antimicrobial agents [26]. The susceptibility of biofilm cells to antimicrobial agents has been shown to differ from that of planktonic cultures [24] and this is a major contributor to the etiology of infectious diseases. In addition, another distinctive property is that biofilm cells exhibited different pattern of gene expression including the expression of virulence factor genes [27]. This property can involve a cell-to-cell communication system called quorum sensing (QS) [28]. The signaling molecules are known as autoinducers (AIs). When these molecules reach a critical threshold concentration, a signal transduction cascade is triggered. Signaling by AIs in the QS system forms the basis for alterations in various gene expressions including virulence factors, secretion system, motility, sporulation, and biofilm formation [29]. Three QS molecules were well characterized (oligopeptides, AI-1, and AI-2). Oligopeptides are produced by gram-positive bacteria and their action is species-specific. Many gram-negative bacteria utilize N-acyl-L-homoserine lactone (N-AHL) molecules as AI-1 signaling molecules [30], and these activities are also species-specific. A wide range of gram-positive and gram-negative bacterial species utilize AI-2 signaling molecules which are furanosyl borate diesters, and the enzyme responsible for their synthesis is encoded by the *luxS* gene [31, 32]. These AI systems play important roles in bacterial biofilm formation.

3. The Properties of *H. pylori* Biofilms

In an initial investigation on biofilm formation by *H. pylori* two studies characterized biofilm formation by this organism [14, 15]. As the first demonstration of the *in vitro* ability to form biofilms by *H. pylori*, Stark et al. reported that a water insoluble polysaccharide-containing biofilm has been observed at the air-liquid interface when *H. pylori* strain NCTC 11637 was continuously grown in a glass fermenter [14]. Subsequently, Cole et al. reported that all of the *H. pylori* strains used in their study, including clinical isolates, laboratory strains, and a mouse-adapted strain, were able to form biofilms on glass surfaces [15]. They also reported that *H. pylori* could form a biofilm only at the air-liquid interface, which is most likely indicative of its microaerobicity. However, at present, biofilm formation by *H. pylori* has not been extensively characterized. Therefore, we analyzed the ability of *H. pylori* strains to form biofilms and characterized the underlying mechanisms involved. Initially, we established a feasible and stable model for biofilm formation by this microorganism. Briefly, sterilized glass coverslips were placed into 12-well microtiter plates. Each well was filled with 2 mL of *Brucella* broth supplemented with 7% fetal calf serum (FCS) to allow adherence of *H. pylori* at the air-liquid interface. The formation of biofilms was initiated by inoculating approximately 5×10^5 cells into each well. The cultures were incubated under microaerobic conditions at 37°C for 3 to 5 days with shaking. Using this model, the biofilm forming ability of eight *H. pylori* strains including standard SS1, ATCC 43579, ATCC 43579, and NCTC11638 strains and clinical isolates from Japanese patients was analyzed. Under these conditions, all of the strains formed biofilms at the liquid-gas interface of the cultures. Specifically, strain TK1402, which was isolated from a Japanese patient with duodenal and gastric ulcers, showed significantly higher levels of biofilm formation relative to the other strains (Figure 1) [33]. The strong biofilm forming ability of TK1402 was reflected in the relative thickness of the biofilms. To clarify the architectural characteristics of *H. pylori* biofilms, we compared TK1402 and SS1 biofilms

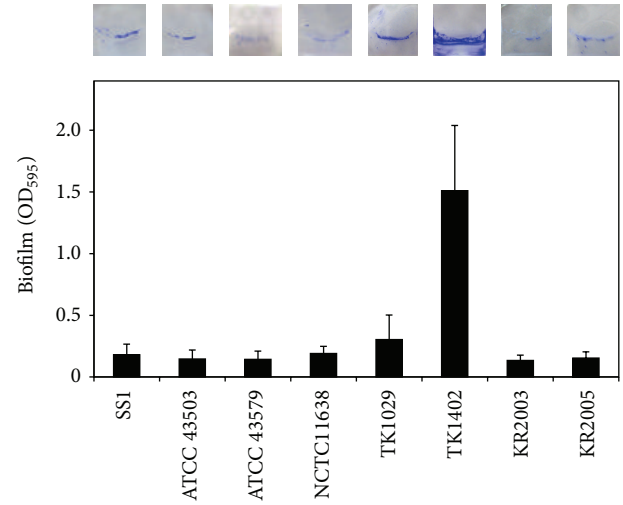

Figure 1: Biofilm formation by *H. pylori* strains. The graph shows quantification of biofilms formed after 3 days following culture in *Brucella* broth supplemented with 7% FCS. The upper photographs show typical biofilms on glass coverslips.

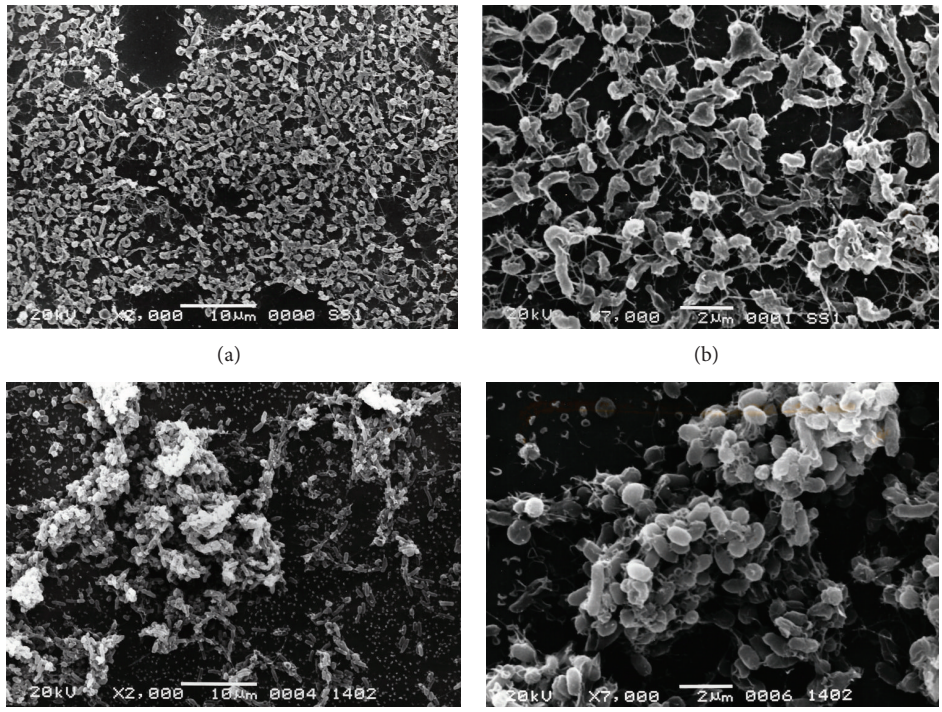

Figure 2: SEM images of *H. pylori* strains SS1 ((a) and (b)) and TK1402 ((c) and (d)) biofilms. The 3-day biofilm of each strain on cover glass was investigated using SEM. Photographs were taken at low (×2000; (a) and (c)) or high (×7000; (b) and (d)) magnification. Scale bar (2 μm) is shown at the bottom of each electron micrograph.

by scanning electron microscopy (SEM) (Figure 2) [34]. In the SS1 biofilms, the bacteria attached to glass surfaces in thin layers, and the biofilms consisted mainly of bleb-like or amorphous structures (Figures 2(a) and 2(b)). On the other hand, the TK1402 biofilms were composed primarily of cells with bacillary morphology which were clearly outlined (Figures 2(c) and 2(d)). We also analyzed the biofilm cells of the other strains using SEM. However, the majority of these biofilm cells consisted of autolysed cells, suggesting that the strong biofilm forming ability of TK402 may have resulted from an active metabolic state for a relatively long time without exhibiting morphological changes or autolysis. In addition, the biofilms of TK1402 strain showed the presence of many outer membrane vesicles (OMVs) on the glass surfaces as well as on the bacterial cell surfaces. These structures were not detected in the biofilms of the other strains. OMVs were more closely observed in the thin-sectioned biofilms using transmission electron microscopy (TEM) and the OMVs were located at the substratum-bacterium interface and in the extracellular spaces. In addition, biofilm formation by strain TK1402 was strongly correlated with the production of OMV. These results suggested that the OMV produced by strain TK1402 may serve as an EPS matrix for these biofilms. OMV production is a physiologically normal function of gram-negative bacteria [35, 36]. In *Pseudomonas aeruginosa*, OMVs have multifunctional biological roles including microbial interaction and host infection as well as maintenance of the structure of biofilm [37, 38]. In *Porphyromonas gingivalis*, OMVs promote attachment, aggregation, and biofilm formation and the functions of OMVs in biofilms have been discussed [39, 40]. Similar to most gram-negative bacteria, *H. pylori* released OMV into the extracellular space [41, 42]. Major protein and phospholipid components associated with the OMVs were identified [43]. We analyzed the protein profile of the OMV produced by strain TK1402 to determine which components of the OMV contribute to biofilm formation in *H. pylori*. The results indicated that a specific approximately 22 kDa protein might be involved in the biofilm forming ability of this strain [44]. Additional research is now in progress to determine what factors are directly involved in biofilm formation by strain TK1402.

Concerning the *H. pylori* biofilm matrix, Grande et al. demonstrated that extracellular DNA is a component of EPS structures and is important in stabilizing biofilm structures [45]. Yang et al. indicated that mannose-related proteoglycans (proteomannans) are one component of the EPS structures and proteomannans are also involved in the process of *H. pylori* biofilm formation [46]. They also reported that the neutrophil-activating protein A (NapA) is upregulated in biofilm cells compared to planktonic cells, and biofilm formation with a *napA* deficient mutant exhibited a different phenotypic biofilm. Recently, Grande et al. demonstrated that biofilms developed by multiple *H. pylori* strains are more complex than those associated with single strains and such conditions might promote genetic exchange favoring the generation of more virulent strains [47].

4. Quorum Sensing in *H. pylori*

The *luxS* gene is the only known quorum-sensing gene present in the sequenced *H. pylori* genome. Several reports indicated that *H. pylori* produces extracellular signaling molecules related to AI-2, and production of AI-2 is dependent on *luxS* function [48–50]. These reports have indicated that the production of AI-2 by *luxS* is growth-phase dependent, with maximal production occurring in the mid-exponential phase of growth. Several reports indicated that LuxS has an alternative role in regulation of motility by modulating flagellar transcription and flagellar biosynthesis [51, 52]. Our previous study also demonstrated that strain TK1402 *luxS* deficient mutant exhibited significantly lower motility than that of parental strain [53]. In addition, the *luxS* mutant exhibited a reduced infection rate relative to the wild-type parent strain TK1402 in a Mongolian gerbil model. Cole et al. reported the relations of *luxS* quorum sensing and biofilm formation in *H. pylori* [15]. They demonstrated that the *luxS* mutants of clinically isolated strains, SD3 and SD4, were approximately twofold more better at forming a biofilm than the parental strains. On the other hand, Doherty et al. indicated that LuxS fulfills primarily a metabolic role in the activated methyl cycle, which generates the *S*-adenosylmethionine required by methyltransferases and recycles the product via methionine as well as cell-to-cell signaling [54]. Further investigations are expected to elucidate the function of LuxS.

5. *H. pylori* Biofilm Formation in the Environment

The principal mode of transmission proposed for *H. pylori* is person to person contact via the faecal-oral, oral-oral, or gastro-oral routes [55–58]. However, especially in developing countries, the patterns of *H. pylori* transmission suggest a universal source for exposure rather than person to person transmission [59]. Thus, the drinking water supply was highlighted as an important source of *H. pylori* infection and, indeed, *H. pylori* was only detected with special procedures in water distribution systems [60, 61]. In addition, the role of water sources and associated biofilms acting as environmental transmitters of *H. pylori* has been suggested by the detection of *H. pylori* DNA by molecular methods, such as PCR, in sewage, well water, pond and river water, river water, and shallow ground water in developed countries as well as in developing countries [61–66]. These data suggested that *H. pylori* exists in water distribution systems and that the organism may survive in biofilms in these systems. However, in fact, it does not appear that *H. pylori* forms biofilms at locations which are relatively stressful conditions such as less than optimal temperatures and nutrient limitation. In oligotrophic water systems, the bacterial genera *Pedomicrobium*, *Hyphomicrobium*, *Gallionella*, and *Caulobacter* were regularly found [67]. It is likely that these bacteria form biofilms in drinking water distribution systems and are then contaminated with *H. pylori* from sewage, well water, pond and river water, river water, and shallow ground water and are embedded in such bacterial biofilm structures. Indeed, *H. pylori* has never been cultured from drinking water distribution systems using standard cultivation techniques [68, 69]. These reports indicated that it is impossible to distinguish between alive and dead cells of *H. pylori* in such systems. Recently, it was reported with several new methods such as in situ fluorescent hybridization (FISH) [20, 70] to detect viable *H. pylori* in various water sources. Continuous critical investigation is necessary as it remains unclear to what extent there is a health risk from this source.

6. *H. pylori* Biofilm Formation on Human Gastric Mucosa

The first photographic documentation of the existence of *H. pylori* biofilms on human gastric mucosa was reported by Carron et al. using endoscopically directed biopsies and scanning electron microscopy [17]. Mature biofilms were present and attached to the cell surface of *H. pylori*-positive specimens. Their group subsequently reported that, among patients with peptic ulcer disease who were tested urease positive for *H. pylori*, the average rate of total cell surfaces covered by biofilms was 97.3%, as opposed to 1.64% for urease-negative patients [18]. Cellini et al. reported that a prevalent S-shape *H. pylori* morphotype which coexisted with coccid aggregated bacteria embedded in an abundant matrix was demonstrated by SEM analysis with biopsies from patients harboring culturable bacteria [19]. On the other hand, samples from patients shown as *H. pylori*-positive only through the molecular methods showed clustered coccid bacteria arranged in a microbial biofilm. Cammarota et al. reported that, among the patients who had a history of at least four *H. pylori* eradication failures, SEM analysis of gastric biopsies showed that *H. pylori* formed biofilms on the gastric mucosa in all of the patients and that the biofilm disappeared in all of them when the microorganism was eradicated [71].

7. Effects of *H. pylori* Biofilms on Susceptibility to Antimicrobial Agents

Eradication of *H. pylori* is important not only for the treatment of gastric/duodenal ulcer, but also for the treatment and prevention of *H. pylori*-associated diseases such as gastric cancer, as well as for inhibiting the spread of this microorganism. For the eradication of *H. pylori*, a combination therapy using an antiacid agent (proton pump inhibitor (PPI) or H_2 blocker) and two anti-*H. pylori* agents (amoxicillin and either clarithromycin (CAM) or metronidazole) has been recommended [72–74]. Fluoroquinolones have also been selected as anti-*H. pylori* agents. In Japan, a combination of a proton pump inhibitor, amoxicillin, and CAM is commonly used in first-line eradication therapy [72]. However, CAM resistance is an increasing problem for the first-line therapy of *H. pylori* infection, since the major cause of eradication failure is thought to be the existence of CAM resistant *H. pylori* [72, 74–77]. CAM resistant *H. pylori* are extremely common and the frequency of CAM resistant clinical isolates ranges from approximately 10 to 30% [74, 78]. Point mutations in the domain V loop of the 23S rRNA gene (commonly an

Table 1: Generation of CAM resistance mutations in biofilm and planktonic cells. The 2-day and 3-day biofilms and planktonic cells were exposed to the indicated concentrations of CAM (biofilms were exposed to one-eighth, one-quarter, or one-half of the MBC of CAM at concentrations of 0.125, 0.25, and 0.5 μg/mL, concentrations which are equivalent to 8x, 16x, and 32x MIC and planktonic cultures were also exposed to one-quarter or one-half of the MBC of CAM at concentrations of 0.063 and 0.125 μg/mL, concentrations which are equivalent to 4x and 8x MIC) for 24 h under microaerobic conditions at 37°C with shaking. After incubation, cells were recovered in fresh *Brucella* supplemented with 7% FCS agar, and the generation of CAM resistant mutants was assessed in media supplemented with 1.0 μg/mL CAM. When no CAM resistant cells were detected, exposure to CAM was repeated up to 5 times. The table indicates the accumulation ratio of the generated CAM resistance in biofilms (number of samples was 12 or 13) or in planktonic cultures (number of samples was 12).

Samples	Passage time				
CAM concentrations	1st	2nd	3rd	4th	5th
2-day biofilm					
CAM 0.5 μg/mL	0/12 (0%)	0/12 (0%)	1/12 (8%)	2/12 (17%)	4/12 (33%)
CAM 0.25 μg/mL	1/12 (8%)	4/12 (33%)	6/12 (50%)	8/12 (67%)	9/12 (75%)
CAM 0.125 μg/mL	0/12 (0%)	1/12 (8%)	2/12 (17%)	3/12 (25%)	4/12 (33%)
2-day planktonic					
CAM 0.125 μg/mL	0/12 (0%)	0/12 (0%)	1/12 (8%)	4/12 (33%)	4/12 (33%)
CAM 0.063 μg/mL	0/12 (0%)	0/12 (0%)	3/12 (25%)	3/12 (25%)	3/12 (25%)
3-day biofilm					
CAM 0.5 μg/mL	1/12 (8%)	3/12 (25%)	4/12 (33%)	6/12 (50%)	6/12 (50%)
CAM 0.25 μg/mL	1/13 (8%)	5/13 (38%)	11/13 (85%)	11/13 (85%)	11/13 (85%)
CAM 0.125 μg/mL	1/13 (8%)	2/13 (15)	3/13 (23%)	5/13 (38%)	6/13 (46%)
3-day planktonic					
CAM 0.125 μg/mL	0/12 (0%)	1/12 (8%)	1/12 (8%)	1/12 (8%)	3/12 (25%)
CAM 0.063 μg/mL	1/12 (8%)	1/12 (8%)	1/12 (8%)	1/12 (8%)	3/12 (25%)

adenine-to-guanine transition at position 2142 or 2143) have been reported as the basis for resistance [72, 74–79].

In other bacterial biofilms, biofilm grown cells express properties distinct from planktonic cells, one of which is an increased resistance to antimicrobial agents [26, 80–83]. Based on these reports, the biofilm cells can become 10–1000 times more resistant to the effects of antimicrobial agents. Multiple mechanisms of biofilm resistance to antimicrobial compounds were suggested: (i) failure of the antimicrobial compounds to penetrate the biofilm, (ii) slow growth of the biofilm cells owing to nutrient limitation, and (iii) activation of the general stress response [26, 84–88]. However, the effect of *H. pylori* biofilm formation on antibiotics susceptibility is not well documented. Thus, we investigated the effects of CAM on *H. pylori* biofilms [89]. Biofilm formation in *H. pylori* increased the resistance to CAM at minimum inhibitory concentration (MIC) levels by up to 4-fold in 2-day biofilms (intermediated biofilms) and to 16-fold in 3-day biofilms (mature biofilms) as well as minimum bactericidal concentration (MBC) levels by up to 4-fold compared to planktonic cells. Participation of the efflux pumps of the resistance-nodulation-cell division (RND) family was involved in the development of CAM resistance in *H. pylori* biofilm and failure of CAM penetration into the biofilm interior due to the presence of the extracellular matrix was also demonstrated. In addition, we demonstrated that *H. pylori* biofilm formation can affect the generation of CAM resistance mutations (Table 1). CAM resistant cells were detected more frequently in biofilms after treatment with CAM. Our results indicated that the relatively high concentration, especially one-quarter of MBC (0.25 μg/mL, which are concentrations equivalent to 16x MIC), of CAM may facilitate the generation of CAM resistance mutations in *H. pylori* biofilms.

8. Therapy for Preventing *H. pylori* Biofilm Infection

Antibiotic resistance in *H. pylori* can therefore be acquired by the selection of spontaneous mutation events that occur due to the magnitude and duration of antibiotic use on the human gastric mucosa. Nakamura et al. reported that CAM concentrations in gastric juices, mucosa, or serum after administration of 500 mg of the drug for 7 days were 550.6, 64.6, and 2.5 μg/mL at 2 hours after administration and 43.4, 36.2, and 2.2 μg/mL at 6 hours, respectively [90]. These concentrations might be sufficient to reduce the levels of *H. pylori in vivo* so that this microorganism formed biofilms. However, to reach such high concentrations of CAM on the gastric mucosa for extended periods, the drug needs to be taken with sufficient dosage. In addition, in cases with inadequate compliance with eradication therapy, the concentration of CAM does not reach high levels in the gastric mucosa. Further, macrolides including CAM are frequently used in the treatment of various infectious diseases in pediatric, respiratory, and otorhinolaryngology settings. In these cases, biofilm formation by *H. pylori* may contribute to the acquisition of CAM resistance.

Novel approaches to prevent biofilm formation and to treat infections by biofilm-forming bacteria are currently under development [91, 92]. Recently, a clinical trial for effective strategies targeting *H. pylori* biofilm infections through the use of molecules such as *N*-acetylcysteine (NAC) was

reported [71, 93]. NAC is a mucolytic and a thiol-containing antioxidant agent and is considered a nonantibiotic drug that has antibacterial properties. In 1977, Parry and Neu found that NAC had the ability to inhibit the growth of both gram-positive and gram-negative bacteria, including *Staphylococcus aureus*, *P. aeruginosa*, *Klebsiella pneumoniae,* and *Enterobacter cloacae* [94]. The antibacterial effect of NAC may be due to competitively inhibiting amino acid (cysteine) utilization or by virtue of possessing a sulfhydryl group it may react with bacterial cell proteins. Moreover, previous studies demonstrated decreased biofilm formation by a variety of bacteria in the presence of NAC [95–98], leading to an inhibition of bacterial adherence, a reduction in the production of the extracellular polysaccharide matrix promoting the disruption of mature biofilms, and a reduction in sessile cell viability [95–98]. Relative to *H. pylori* biofilms, NAC is effective in both inhibiting *H. pylori* biofilm formation and disrupting developed biofilms *in vitro* [71]. In addition, NAC treatment preceding the initiation of antibiotic eradication therapy is able to provide eradication of resistant *H. pylori* infections. Large scale studies regarding the effectiveness of NAC *in vivo* for reducing *H. pylori* biofilms are still required.

9. Conclusions

Pathogenic bacteria including *H. pylori* within biofilms can escape from both host immune responses and the effects of antimicrobial agents. Consequently, chronic infections by biofilm forming bacteria become troublesome and difficult to treat. Some of the previous studies have shown that *H. pylori* forms biofilm on human gastric mucosa. Nevertheless, assessment of *H. pylori* strain susceptibility to antibiotics *in vitro* has traditionally been evaluated using planktonic cells, so that MICs are not reliable predictors of the antibiotic effects in the human stomach. The assessment of the ability to form biofilms in *H. pylori* could play an important role in preventing and controlling the generation of antibiotic resistance. It is expected that enhancing our knowledge of *H. pylori* biofilm formation will lead to new treatment therapies for preventing *H. pylori* infections. However, it is recognized that our understanding of *H. pylori* biofilm formation is still in its infancy. Further studies of the mechanism of *H. pylori* biofilm formation need to be performed. In addition, investigation into novel *H. pylori* eradication strategies for the human gastric mucosa using biofilm-dissolving compounds, quorum sensing inhibitors, or conventional antibiotics may provide advantages in resolving *H. pylori* infections.

Conflict of Interests

The authors have declared that no competing interests exist.

Acknowledgments

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Clinical Study

Seven-Day Nonbismuth Containing Quadruple Therapy Could Achieve a Grade "A" Success Rate for First-Line *Helicobacter pylori* Eradication

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This prospective study was to assess the efficacy of nonbismuth containing quadruple therapy as first-line *H. pylori* treatment and to determine the clinical factors influencing patient outcome. We enrolled 200 *H. pylori*-infected naïve patients. They were prescribed either a 7-day nonbismuth containing quadruple therapy group (EACM, esomeprazole 40 mg twice daily, amoxicillin 1 g twice daily, metronidazole 500 mg twice daily, and clarithromycin 500 mg twice daily) or a 7-day standard triple therapy group (EAC, esomeprazole 40 mg twice daily, amoxicillin 1 g twice daily, and clarithromycin 500 mg twice daily). Follow-up studies to assess treatment responses were carried out 8 weeks later. The eradication rates attained by EACM and EAC groups were 95.6% (95% confidence interval [CI] = 89.4%–98.3%) and 79.3% (95% CI = 70%–86.4%) in the per-protocol analysis ($P < 0.001$) and 88% (95% CI = 80.2%–93.0%) and 73% (95% I = 63.6%–80.3%) in the intention-to-treat analysis ($P = 0.007$). Clarithromycin resistance, metronidazole resistance, and dual clarithromycin and metronidazole resistances were the clinical factors influencing *H. pylori* eradication in EACM group. Clarithromycin resistance and dual clarithromycin and metronidazole resistances were the influential factor for EAC treatment. In conclusion, the results suggest that 7-day nonbismuth containing quadruple therapy could achieve a grade "A" report card for first-line *H. pylori* treatment.

1. Introduction

The Maastricht IV/Florence-Consensus Report recommended that the standard triple therapy should now be avoided in areas where clarithromycin resistance is high (>15–20%) [1]. The reported local primary resistance rate to clarithromycin in Taiwan ranged from 6 to 18% over time [2–7]. However, Chen et al. recently reported the primary resistance rates of metronidazole (52.8% versus 47.7%) and clarithromycin (13.9% versus 22.7%) in patients who lived in urban and rural areas of eastern Taiwan [8]. The bismuth containing quadruple therapy with 10-day duration could be the alternative first-line treatment, especially in areas with a high prevalence of clarithromycin and metronidazole resistance, because of its ability to overcome metronidazole resistance and achieve an eradication rate > 90% [9–11]. Other alternatives include sequential therapy, concomitant therapy, and hybrid therapy, which provide > or close to 90% eradication rates even in

areas with high rates of clarithromycin and metronidazole resistance. However, there were inconsistent reports for the eradication rates of sequential therapy. For instance, Wu et al. [2] and Tsay et al. [12] both reported >90% intention-to-treat (ITT) and per-protocol (PP) eradication rates in Taiwan. In Korea, the reported PP eradication rate of sequential therapy was 85.7% without at least grade "B" report card in 2008 [13]. This year, there were two publications from Korea and China which reported a decrease in 10-day sequential therapy which was only 72.1% by ITT and 78.4% by PP analysis for the Korean report [14] and 72.1% by ITT and 76.5% by PP analysis for the Chinese report [15]. The bottom line is that the inconsistent results may imply that the efficacy of sequential therapy varies in different countries and may have declined over time. It might not be a perfect option in the first-line *H. pylori* eradication anymore, particularly in areas where clarithromycin resistance is high (>15–20%).

One of the other alternatives includes hybrid therapy, which could provide >95% eradication rate even in areas with high rates of clarithromycin and metronidazole resistance [16]. Hsu et al. reported near 100% PP eradication rates for 14-day hybrid therapy [16]. It has two phases: dual therapy with a PPI (standard dose, b.i.d.) and amoxicillin (1 g, b.i.d.) for 7 days, followed by a nonbismuth quadruple therapy consisting of PPI (standard dose, b.i.d.), amoxicillin (1 g, b.i.d.), clarithromycin (500 mg, b.i.d.), and metronidazole (500 mg, b.i.d.) for further 7 days. The benefit of the extended duration of amoxicillin administration is to further reduce the bacterial load to improve the eradication rate but then again it involves very complex regimens [16]. However, recently published reports from Korea and Iran reported eradication rates of 85.9% and 92.9% [17, 18]. It may need more studies to prove its efficacies in different countries and taking into account the local antibiotics resistance to metronidazole and clarithromycin. Besides, the complexity and lengthy duration of the prescription may be the disadvantage.

Concomitant therapy consists of a PPI (standard dose, b.i.d.) combined with clarithromycin (500 mg, b.i.d.), amoxicillin (1 g, b.i.d.), and metronidazole (500 mg, b.i.d.), prescribed all together at the same time which is more convenient than sequential therapy and hybrid therapy because of the shorter duration of treatment and less complex drug administration. As a matter of fact, the concomitant therapy was more convenient and achieves equally efficient eradication rates and is with consistent results over years. Studies with 10-day concomitant therapy achieved an efficacy of > or close to 90% for *H. pylori* eradication rates [2, 19–24]. The current study explored the influential role of 7-day nonbismuth containing quadruple therapy for first-line *H. pylori* eradication to determine which clinical factors influence patient outcome.

2. Materials and Methods

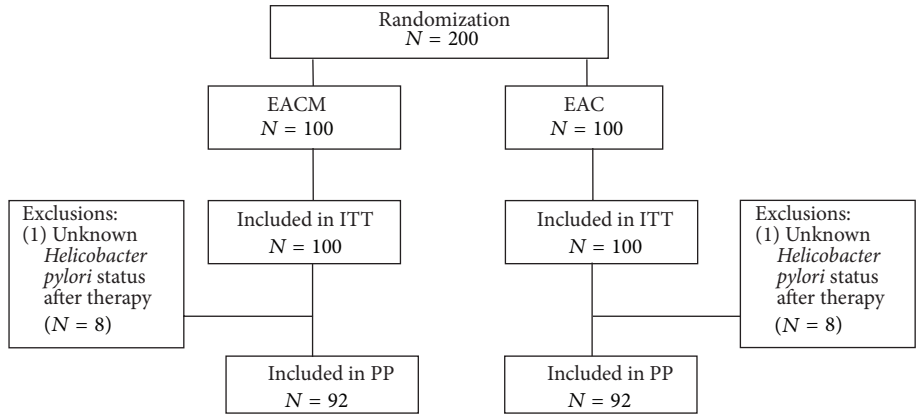
2.1. Patients. From August 2012 to March 2014, a total of 200 *H. pylori*-infected naïve patients were enrolled. All patients were at least 18 years of age and had received endoscope examinations that showed peptic ulcers or gastritis. They were randomly prescribed either a 7-day nonbismuth containing quadruple therapy group (EACM, esomeprazole 40 mg twice daily, amoxicillin 1 g twice daily, metronidazole 500 mg twice daily, and clarithromycin 500 mg twice daily for 7 days) or a 7-day standard triple therapy group (EAC, esomeprazole 40 mg twice daily, amoxicillin 1 g twice daily, and clarithromycin 500 mg twice daily, for 7 days) by their clinicians.

Criteria for exclusion included the following: (1) previous surgery of the stomach, such as partial gastrectomy; (2) use of antibiotics within the preceding 30 days; (3) regular use of a PPI or bismuth compounds (>3 times per week) in the 30 days before enrollment; (4) presence of serious medical condition(s) precluding participation or endoscopy with biopsy; (5) patients previously treated for *H. pylori* infection; (6) use of concomitant medication(s) known to interact with study medication (simvastatin was permitted); (7) presence of Zollinger-Ellison syndrome; (8) pregnancy or lactation; (9) allergy to any medication in the study; (10) contraindication(s) to the use of any of the study drugs; (11) participating in any clinical trial within the last 30 days; (12) unwillingness to abstain from alcoholic beverages; and (13) patients taking other medications including antipsychotics, and chronic nonsteroidal anti-inflammatory drugs were also excluded.

This study was approved by both the Institutional Review Board and the Ethics Committee of Chang Gung Memorial Hospital (IRB-101-0674A3). All patients provided their written informed consent before undergoing endoscopic interventions.

2.2. Outcomes and Follow-Up. The primary endpoint was the successful eradication of *H. pylori*. We also analyzed antibiotic susceptibility. The confirmation of *H. pylori* eradication failure was defined as positive results both for the rapid urease test and by histology after first-line eradication therapy. All registered patients were followed up at week 2 to assess drug compliance and adverse effects after they finished the medication regimens. Drug compliance was assessed via pill counts. Poor compliance was defined as failure to finish 80% of all medication due to adverse effects [25–28]. These patients underwent either an endoscopy or a urea breath test 8 weeks later. We also performed a back-up urea breath test on all participants to avoid any false-negative results.

2.3. Questionnaire. The questionnaire contained questions regarding personal history of smoking and alcohol drinking. The questionnaire was locally derived and not a validated or previously published quality of life questionnaire. Quality of life was not assessed. Smokers were defined as those who consumed more than 1 pack of cigarettes a week, and drinkers were those who drank more than 1 cup of alcoholic beverage per day. The adverse events evaluated included abdominal pain, diarrhea, constipation, dizziness, taste perversion, headache, anorexia, nausea, vomiting, and skin rash. Those who considered those symptoms disturbed their daily life were defined to have major adverse effects. Those who experienced these symptoms but did not consider them a disturbance to their daily life were defined to have minor adverse effects.



EACM group: 7-day esomeprazole/amoxicillin/clarithromycin/metronidazole therapy

EAC group: 7-day standard esomeprazole/amoxicillin/clarithromycin triple therapy

ITT: Intention-to-treat

PP: Per-protocol

Figure 1: Disposition of patients.

2.4. Culture and Antimicrobial Resistance. One antral gastric specimen and one corpus biopsy specimen were obtained for *H. pylori* isolation using previously described culture methods [29]. The biopsy specimens were cultured on plates containing *Brucella* chocolate agar with 7% sheep blood and incubated for 4-5 days under microaerobic conditions. The minimal inhibitory concentration (MIC) was determined by the agar dilution test. *H. pylori* strains with MIC values ≥0.5, ≥1, ≥1, ≥4, and ≥8 mg/L were considered to be the resistant breakpoints for amoxicillin, clarithromycin, levofloxacin, tetracycline, and metronidazole, respectively [2, 3, 16, 25].

2.5. Randomization. A computer-generated randomization list was used to generate a "random sequence." We used a method combining blocking and stratified randomization to ensure a close balance of the numbers and patients' characteristics in each group. We set separate randomization within each of two groups of participants. We then set a block of every 10 participants. A computer-generated randomization list was drawn up by the statistician and given to the physician for randomization. Doctors determined patients' suitability to be enrolled in this study and allocated the next available number on entry into the trial. The code was revealed to the researchers once recruitment, data collection, and laboratory analyses were complete. An independent research assistant generated the computerized random number sequence. The sequence was concealed in an opaque envelope until the intervention was assigned. After the written informed consents were obtained from the participants, an independent research assistant assigned the therapies according to the treatment allocations kept in the envelopes. Each patient collected the medications on the same day from the pharmacy department in our hospital.

2.6. Statistical Analysis. The primary outcome variables were the eradication rate, presence of adverse events, and level of patient compliance. Using the SPSS program (Statistical Package for the Social Sciences version 18, Chicago, IL, USA), Chi-square tests with or without Yates' correction for continuity and Fisher's exact tests were used when appropriate to compare the major outcomes between groups. Eradication rates were analyzed by both ITT and per-protocol (PP) approaches. ITT analysis included all assigned patients who had taken at least one dose of the study medication. Patients whose infection status was unknown following treatment were considered treatment failures for the purposes of the ITT analysis. The PP analysis excluded patients with unknown *H. pylori* status following therapy and those with major protocol violations. A P value <0.05 was considered statistically significant. To determine the independent factors that affected treatment response, the clinical and bacterial parameters were analyzed by univariate and multivariate analyses.

3. Results

Figure 1 shows patient flowchart, according to the CONSORT statement advice. A total of 200 patients with positive *H. pylori* were recruited into the study and were randomly assigned to receive EACM and EAC therapy. The two treatment groups were matched with respect to baseline demographic data, clinical characteristics, and antibiotic resistance (Table 1). A total of 16 patients were excluded from the PP analysis (8 in each group), because of being lost to follow-up, resulting in 92 in the PP study for each of EACM and EAC groups. The eradication rates in the EAC and EACM groups are detailed in Table 2. They were 95.6% (95% confidence interval [CI] = 89.4–98.3%) and 79.3% (95% CI = 70%–86.7%), respectively, in the PP analysis ($P < 0.001$); 88% (95% CI = 80.2%–93%) and 73% (95% CI = 63.6%–80.3%), respectively, in the ITT analysis.

Table 1: Demographic data and endoscopic appearances of the two patient groups.

Characteristics	EACM ($n = 92$)	EAC ($n = 92$)	P value
Age (year) (mean ± SD)	47.8 ± 11.6	52.8 ± 12.8	0.593
Gender (male/female)	45/47	46/46	0.883
Smoking	14	14	1.000
Alcohol consumption	20	14	0.254
Previous history of peptic ulcer	14	19	0.337
Endoscopic findings			0.964
Gastritis	32	34	
Gastric ulcer	22	22	
Duodenal ulcer	31	28	
Gastric and duodenal ulcer	7	8	

EACM group: 7-day esomeprazole/amoxicillin/clarithromycin/metronidazole therapy; EAC group: 7-day standard esomeprazole/amoxicillin/clarithromycin/ triple therapy.

Table 2: Major outcomes of eradication therapy.

	Eradication rate		
	EACM ($n = 92$)	EAC ($n = 92$)	P value
Intention-to-treat	88% (88/100)	73% (73/100)	0.007
Per-protocol	95.6% (88/92)	79.3% (73/92)	<0.001
Adverse event	30.4% (28/92)	16.3% (15/92)	0.024
Compliance	100% (92/92)	100% (92/92)	1.000

EACM group: 7-day esomeprazole/amoxicillin/clarithromycin/metronidazole therapy; EAC group: 7-day standard esomeprazole/amoxicillin/clarithromycin/ triple therapy.

3.1. Adverse Events and Complications. The adverse event rates were 30.4% (28/92) in EACM group and 16.3% (15/92) in EAC group, $P = 0.024$ (Table 3). These adverse events included abdominal pain, constipation, diarrhea, dizziness, headache, and nausea/vomiting; there were more patients in the EACM group experiencing nausea sensation than the EAC group (30.4% versus 16.3%, $P = 0.024$). However, these were mild and did not markedly disturb the patients' daily activities and importantly both groups had good drug compliances (100% in all of them).

3.2. Antibiotic Resistance. Samples from 90 patients were cultured for *H. pylori*, and the positive culture rate was 75.6% (68/90). The results of the *H. pylori* eradication rate for different subgroups susceptible to amoxicillin, clarythromycin, and metronidazole for both EAC and EACM patients are found in Table 4.

3.3. Factors Influencing the Efficacy of Anti-H. pylori Therapies. Univariate analysis for factors that influence the efficacy was shown in Table 4. Clarithromycin resistances (CLA-R) were significant factors in both the EACM group ($P = 0.002$) and the EAC group ($P < 0.001$). Metronidazole resistance (MET-R) is the risk factors for eradication failure in the EACM group ($P = 0.009$) but not the EAC group. However, dual resistances to both antibiotics were the risk factors for eradication failure in both EACM and EAC groups ($P < 0.001$).

4. Discussion

Our data confirm that the previously reported efficacy of using concomitant therapy achieves an efficacy of > or close to 90% for *H. pylori* eradication rates but we achieved a grade "A" PP result (95.6%) with 7-day regimen instead of 10-day one [2, 21–24]. These findings merit the recommendations of concomitant therapy as the optimal formula in addition to the advantage of being far more easy and convenient for patients to follow than the sequential and hybrid therapies.

Possible explanations for the discrepancies in the reports for both sequential and hybrid therapies as first-line *H. pylori* eradication regimens are different antibiotic resistances of *H. pylori* strains and different treatment durations. Besides the regional variations in eradication efficacies, sequential and hybrid therapy are much more complex in terms of medication requirements because the patients need to switch drugs during the treatment course. This might result in reducing patient compliance and physician preference to prescribe the regimen [16–18, 30]. Therefore, concomitant therapy might be a relatively optimal treatment option in terms of convenience and consistent efficacies among the three. Nevertheless, the inevitable problematic issue is that CLA-R and MET-R could be crucial in the efficacy of *H. pylori* eradication [31].

Currently, the decrease in the first-line *H. pylori* eradication rates in standard 7-day triple therapy is relevant to the progressively increasing CLA-R resistance rates in many parts of the world, particularly those areas with the CLA-R above

Table 3: Adverse events during eradication therapies.

Adverse event	EACM (n = 92)	EAC (n = 92)	P value
Abdominal pain	9 (9.8%)	6 (6.5%)	0.419
Constipation	1 (1.1%)	2 (2.2%)	0.560
Diarrhea	11 (11.9%)	6 (6.5%)	0.203
Dizziness	7 (7.6%)	4 (4.3%)	0.315
Headache	6 (6.5%)	2 (2.2%)	0.148
Nausea/vomiting	10 (10.8%)	2 (2.2%)	0.017
Skin rash	0 (0%)	0 (0%)	—
Taste perversion	0 (0%)	0 (0%)	—

EACM group: 7-day esomeprazole/amoxicillin/clarithromycin/metronidazole therapy; EAC group: 7-day standard esomeprazole/amoxicillin/clarithromycin/ triple therapy.

Table 4: Univariate analysis of the clinical factors influencing the efficacy of *H. pylori* eradication.

Principle parameter		EACM			EAC		
		Case number	Eradication rate (%)	P value	Case number	Eradication rate (%)	P value
Age	<60 years	75/78	96.1	0.578	52/64	81.2	0.496
	≥60 years	13/14	92.8		21/28	75.0	
Sex	Female	44/47	93.6	0.328	33/46	71.1	0.071
	Male	44/45	97.8		40/46	86.9	
Smoking	(−)	74/78	94.9	0.386	61/78	78.2	0.523
	(+)	14/14	100.0		12/14	85.7	
Alcohol consumption	(−)	69/72	95.8	0.872	61/78	78.2	0.523
	(+)	19/20	95.0		12/14	85.7	
Previous history of peptic ulcer	(−)	74/78	94.9	0.386	57/73	78.1	0.557
	(+)	14/14	100.0		16/19	84.2	
Compliance	Good	92/92	100.0	—	92/92	100.0	—
	Poor	0	0		0	0	
		H. pylori culture (n = 68)					
Amoxicillin	Susceptible	31/34	91.2	—	15/33	45.4	0.367
	Resistance	0	—		0/1	0	
Clarithromycin	Susceptible	29/30	96.7	0.002	15/15	100	<0.001
	Resistance	2/4	50.0		0/19	0	
Metronidazole	Susceptible	23/23	100	0.009	11/24	45.8	0.755
	Resistance	8/11	72.7		4/10	40.0	
Dual resistance	Absent	30/31	96.8	<0.001	15/28	53.6	<0.001
	Present	1/3	33.3		0/6	0	

EACM group: 7-day esomeprazole/amoxicillin/clarithromycin/metronidazole therapy; EAC group: 7-day standard esomeprazole/amoxicillin/clarithromycin triple therapy.

20% [30]. The current study showed eradication rates of 73% in ITT and 79.3% in the PP analysis in EAC group. Univariate and multivariate analyses of our data identified CLA-R as a factor that reduced the efficacy of concomitant therapy. None of our 19 patients with CLA-R assigned to EAC were eradicated and the same occurred to the other six patients with dual resistance (CLA-R and MET-R) as shown in Table 4. This was the same for EACM group. Two out of four CLA-R patients in the EACM group were eradicated (50%) while the eradication rates dropped to 33.3% when dual resistance was present. These findings implied that dual resistance could be a major factor affecting the outcome of both EACM and EAC patients. The bottom line is that the prevalence of dual resistance in our study was only 13.2% among the PP population (9/68) and the low number of patients makes the possibility of a type II error likely. For instance, the majority of dual resistances that had treatment failure in EAC arm were due to clarithromycin resistance as demonstrated by the fact that none of the 19 clarithromycin resistance strains were successfully eradicated while 4/10 of metronidazole resistance strains were still successfully eradicated under the EAC arm. Therefore, we should be careful during interpretation of these data. It would be overestimated by drawing conclusion that dual clarithromycin and metronidazole resistance influences the treatment outcome of EAC arm. The effect could be likely due to clarithromycin resistance alone.

When bismuth quadruple therapy is not available, non-bismuth containing quadruple therapy (concomitant therapy) is recommended in high prevalence of CLA-R areas by the Maastricht IV Consensus Report [1]. As mentioned earlier in this text, the reported local primary resistance rate to clarithromycin in Taiwan is increasing over time [2–8]. It was therefore suggested that standard seven-day triple therapy is not suitable for patients in Eastern Taiwan because the *H.*

pylori eradication rates were only 57.5% by ITT analysis and 61.8% by PP analysis. This is the same in current study as we attained only an eradication rate of 79.3% by PP analysis and 73% by ITT analysis. Both these Taiwanese studies, including ours, suggested that the clinical practice for first-line eradication with standard triple therapy should be abandoned. However, for patients who encountered dual resistances, although 7-day EACM eradication rates are higher than EAC group (33.3% versus 0%, $P = 0.134$), it implies that in areas with high prevalence of CLA-R even concomitant therapy may not be suitable to eradicate patients with CLA-R and MET-R resistances. The impact of MET-R on the outcome of concomitant therapy is relatively less marked than CLA-R. Data on *H. pylori* eradication with concomitant therapy in isolated MET-R populations from previously published studies indicate that eradication rates are >85% [2, 23], which is similar to our own findings (72.7% in the EACM group versus 40% in the EAC group).

This study encounters several limitations. Firstly, the sample size is clearly insufficient for a treatment comparison. An adequate sample for reliably detecting clinically significant differences between the two arms would be much larger than the number of patients included in the study. Therefore this study should be considered to be a pilot study that reports preliminary results on the two first-line therapies rather than being treated as a comparative trial. Secondly, the small populations with antibiotic resistance data included in this study have impeded the evaluation of the effects of antibiotic resistance on eradication efficacy. Large sample sized, prospective randomized studies are mandatory to precisely interpret the association of antibiotic resistance to the efficacy of concomitant therapy in Taiwan. Up to now, standard triple therapy was still recommended by the Taiwanese National Health Insurance Administration as the first-line empiric regimen but the overall eradication rates have dropped to <80% as shown in current study. We believe that our results will help facilitate the selection of appropriate patients for concomitant therapy in Taiwan because the more significant rise in clarithromycin resistance is unavoidable for Taiwanese in the near future. It is therefore likely that eradication rates will continue to drop.

5. Conclusion

In conclusion, the results suggest that a 7-day nonbismuth containing quadruple therapy could achieve a grade "A" report card (>95% eradication rate) for first-line *H. pylori* treatment.

Disclosure

Wei-Chen Tai and Chih-Ming Liang are co-first authors.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Helicobacteraceae in Bulk Tank Milk of Dairy Herds from Northern Italy

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Helicobacter pylori is responsible for gastritis and gastric adenocarcinoma in humans, but the routes of transmission of this bacterium have not been clearly defined. Few studies led to supposing that *H. pylori* could be transmitted through raw milk, and no one investigated the presence of other Helicobacteraceae in milk. In the current work, the presence of Helicobacteraceae was investigated in the bulk tank milk of dairy cattle herds located in northern Italy both by direct plating onto *H. pylori* selective medium and by screening PCR for Helicobacteraceae, followed by specific PCRs for *H. pylori*, *Wolinella* spp., and "*Candidatus Helicobacter bovis*." Three out of 163 bulk milk samples tested positive for Helicobacteraceae, but not for the subsequent PCRs. *H. pylori* was not isolated in any case. However, given similar growth conditions, *Arcobacter butzleri, A. cryaerophilus*, and *A. skirrowii* were recovered. In conclusion, the prevalence of Helicobacteraceae in raw milk was negligible (1.8%), and *H. pylori* was not identified in any of the positive samples, suggesting that, at least in the farming conditions of the investigated area, bovine milk does not represent a potential source of infection.

1. Introduction

Helicobacter pylori colonizes the stomach of approximately one half of the world population and is involved in the pathogenesis of several diseases, such as chronic gastritis, peptic ulcers, gastric adenocarcinoma, and mucosa-associated lymphoid tissue tumours [1]. The routes by which *H. pylori* is transmitted have not been firmly established, but different studies support the direct oral-oral transfer or the indirect faecal-oral transmission [2, 3]. Recently, some studies suggested that *H. pylori* can colonize the gastrointestinal tract of domestic ruminants, which could act as reservoirs and transmit the microorganism through contaminated milk. This was firstly proposed by Dore and colleagues [4], who detected the DNA of *H. pylori* in 60.3% of individual sheep milk and were able to culture the bacterium from one sample. Similarly, in Japan, Fujimura et al. [5] demonstrated the presence of *H. pylori* DNA in 72% of raw and 55% of pasteurized bovine milk and isolated one strain. Later on, in Southern Italy, Quaglia and coworkers reported a prevalence of *H. pylori* of 50%, 25.6%, and 33% in bovine, ovine, and caprine bulk milk [6] and of 6% in sheep gastric mucosa [7], but no isolates were obtained after culture of the PCR-positive samples. Also Angelidis et al. [8] detected *H. pylori* in 20% of bovine bulk tank milk by fluorescence *in situ* hybridization, while Safaei et al. [9] reported a prevalence of 16 and 40%, respectively, in individual milk and bovine feces based on PCR and antigen detection tests. On the contrary, both Jiang and Doyle [10], and Turutoglu and Mudul [11] failed to detect *H. pylori* in bovine and ovine raw milk in the US and in Turkey, respectively, by PCR and bacteriological analysis, so the hypothesis that *H. pylori* is transmitted with contaminated milk is still debated.

With the exception of *H. pylori*, no investigation on the presence of other Helicobacteraceae in the milk has been so

far performed and very few studies researched Helicobacteraceae in gastrointestinal tract of cattle. *Wolinella succinogenes*, which belongs to the family Helicobacteraceae, was originally isolated from cattle rumen [12], and "*Candidatus Helicobacter bovis*" was described in the pyloric portion of the abomasum [13]. The study of Helicobacteraceae other than *H. pylori* in animals is valuable because some *Helicobacter* spp. (e.g., *H. heilmannii*, *H. suis*, *H. felis*, and *H. pullorum*) have a zoonotic potential and are responsible for gastrointestinal disorders, and rarely bacteremia in humans [14].

The aim of this study was to investigate the prevalence of *H. pylori* and other Helicobacteraceae in raw milk of dairy cattle of an intensive farming area in order to assess the potential zoonotic role of milk in the transmission to humans of these bacteria, especially because in this area the consumption of raw milk purchased by self-service automatic vending machines represents a common practice.

2. Material and Methods

2.1. Sampling. Milk samples were collected between September and December 2013 from the bulk milk tanks of 163 dairy herds of Lodi Province (located in northern Italy) undergoing the routine monitoring programs. In this area, the average herd size was 150 milking cows and annual milk production per animal 9,000 kg. The milk samples were transported chilled to the laboratory and processed within 6 hours after the collection.

2.2. Microbiological Analysis. A 50 μL aliquot of the samples was streaked onto *H. pylori* selective medium, containing Columbia Blood Agar base, 7% laked horse blood, and DENT Supplement (all from Oxoid, Ltd., Basingstoke, United Kingdom), prepared according to the manufacturer's instructions. The plates were incubated for seven days at 37°C in a microaerophilic atmosphere (GENbox; bioMérieux, Marcy l'Etoile, France). Colonies with typical morphology (small or very small, round, or translucent colonies) were subcultured and subjected to Gram staining. Gram-negative spiral-shaped rods were subjected to species identification by molecular analysis.

2.3. Molecular Analysis. DNA was extracted from milk samples slightly modifying the method reported by Graber and colleagues [15]. This protocol should ensure both the release of intracellular or cellular-adhered bacteria and the detachment of bacteria stuck to the fat globules. Briefly, 625 μL of Triton X-100, 312.5 μL of 1% trypsin solution, and 375 μL of *Lactobacillus casei* ($4{\cdot}10^{10}$ CFU) were added to 1 mL of each milk sample. The specimens were incubated at 55°C for 15 min and centrifuged for 15 min at 4000 g. Afterwards, the supernatant was discarded, the pellet was washed with 1 mL of 1x phosphate buffered saline, and the DNA was extracted using the PureLink Genomic DNA Mini Kit (Life Technologies, Paisley, United Kingdom).

The 16S rRNA gene of members of the family Helicobacteraceae was amplified by PCR using primers C97-C98 [16], followed by a nested PCR using the internal pair of primers HelF-HelR2 [17]. Samples positive for Helicobacteraceae were confirmed as positive with primers O68 and M86 [18] targeting the 23S rRNA *Helicobacter* gene and further tested for the presence of *H. pylori*, *Wolinella* spp., and "*Candidatus Helicobacter bovis*" using the PCR method described by Quaglia et al. [6], Craven et al. [19], and De Groote et al. [13], respectively.

H. pylori ATCC 43504 and *W. succinogenes* LMG 7466 were used as positive controls in the respective specific PCRs and in the PCR for Helicobacteraceae. Due to uncultivability of "*Candidatus Helicobacter bovis*" a reference strain is not available; thus the specific PCR was applied to the DNA isolated from the abomasum of an infected cow [20], and PCR products were sequenced with the same primers used for amplification. Comparison of the sequence to the NCBI database through the algorithm BLAST (http://www.ncbi.nlm.nih.gov/BLAST) revealed a 99% similarity with partial sequence of 16S rRNA gene of "*Candidatus Helicobacter bovis*" (accession number: AF317470.1, AF127027.1). This DNA served as positive control in the subsequent PCR reactions and in the PCR for Helicobacteraceae.

Species identification of the cultured strains was performed by 16S rRNA gene sequencing. Sequencing reaction setup was performed in a final volume of 10 μL with Big Dye Terminator kit v 3.1 Chemistry (Applied Biosystems, Paisley, United Kingdom) following protocol instruction. Capillary electrophoresis was performed on ABI 3730 DNA Analyzers (Applied Biosystems). DNA sequences were analyzed with the software BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html); for each positive PCR, consensus sequences were obtained with the algorithm CAP (Contig Assembly Program) [21]; the identity of the 16S rRNA gene sequences was verified by comparison of the consensus sequences to the NCBI database through the algorithm BLAST.

3. Results and Discussion

An overview of the results is shown in Table 1. Three out of 163 bulk tank milk samples tested positive by PCR for Helicobacteraceae (1.8%, CI 95%: 0–3.9%). However these three samples reacted negatively for any of the subsequent species-specific PCRs and no *Helicobacter* spp. were cultured. In four Helicobacteraceae PCR-negative cases a variable number (10–100) of colonies with morphology referable to *Helicobacter* were cultured. Gram staining showed spiral-shaped rods, but species identification revealed that the bacterial isolates were *Arcobacter butzleri* ($n = 2$), *Arcobacter cryaerophilus* ($n = 1$), and *Arcobacter skirrowii* ($n = 1$).

H. pylori is involved in several human diseases, but the routes by which the pathogen is transmitted have not been fully determined, although oral-oral and fecal-oral transmission are usually suspected [3]. By molecular analysis, some surveys reported a high prevalence of *H. pylori* in raw or pasteurized bovine milk [6, 8], but only very few cases of successful isolation of the strain were described [4, 5]. Conversely, other researches failed to detect the pathogen in milk [10, 11]. In the current study, 163 bovine bulk milk samples were assayed for the presence of *H. pylori* both by direct plating onto selective medium and by PCR, but the bacterium

Table 1: Results of molecular and bacteriological analysis performed on 163 bulk tank milk samples.

Number of milk samples	PCR					Bacteriological analysis[b]
	Helicobacteraceae		*H. pylori*[a]	"*Candidatus Helicobacter bovis*"[a]	*Wolinella* spp.[a]	
	16S rRNA	23S rRNA[a]				
156	−	nd	nd	nd	nd	−
3	+	+	−	−	−	−
2	−	+	nd	nd	nd	*Arcobacter butzleri*
1	−	+	nd	nd	nd	*Arcobacter cryaerophilus*
1	−	+	nd	nd	nd	*Arcobacter skirrowii*

[a]PCRs for 23S rRNA gene of *Helicobacter*, *H. pylori*, "*Candidatus Helicobacter bovis,*" and *Wolinella* spp. were performed on samples positive for the screening PCR for 16S rRNA gene of Helicobacteraceae. [b]Strain identification was performed by 16S rRNA gene sequencing (nd: not done).

was not detected in any of the samples. The wide difference between the reported prevalences might be due to the different methods of analysis. We cultured a low volume of milk in order to restrict the overgrowth of contaminating microflora. Unlike a previous study [22], an enrichment process was not applied, because this medium contains nalidixic acid, to which different *Helicobacter* spp. were susceptible [23], and we were interested in culturing not only *H. pylori*, but also other *Helicobacter* species. Moreover, since our samples were transferred chilled to the laboratory, a conversion of *H. pylori* cells to viable but nonculturable (VBNC) forms might have occurred and should be considered responsible for the failed isolation of *H. pylori* from the examined samples, even though different studies demonstrated that *H. pylori* may survive for some days in milk under refrigeration [24–26]. More likely, the difference in reported prevalences can be related to the different geographic areas or farming conditions (i.e., animal-human promiscuity, contemporary housing of cows and small ruminants, and hygienic standards) where the investigations were performed. Moreover, due to the high prevalence of *H. pylori*-infected people, it cannot be excluded that milk contamination occurred during the milking process through carrier milkers.

In the present study three samples tested positive for Helicobacteraceae PCR. As "*Candidatus Helicobacter bovis*" is present at high levels in cattle abomasa [13], one might expect a transit into the intestinal tract and fecal excretion. A similar situation is expected for *Wolinella* spp., which are found in cattle rumen [12]. Personal observations confirm that such bacteria can be frequently found in the gastrointestinal tract of cattle of our region [20]. Since the presence of microorganisms in bulk milk seems to be mainly associated with fecal contamination [27], we evaluated the presence of these two bacteria species in the three milk samples positive for Helicobacteraceae. "*Candidatus Helicobacter bovis*" and *Wolinella* spp. were not detected in any of the samples, suggesting that other Helicobacteraceae can be found in the milk.

No *Helicobacter* spp. were found by bacteriological analysis. This could be due to the overgrowth of contaminating microflora, which prevents the identification of the small colonies referable to *H. pylori*, or to the presence of VBNC forms. Another reason could be the higher sensitivity of the molecular analysis with respect to culture method, which allows also the detection of low number of *Helicobacter* cells in milk samples. Furthermore, some *Helicobacter* species, including "*Candidatus Helicobacter bovis*," are yet uncultivable in common culture media. However, given similar growth conditions, *Arcobacter* spp. were isolated in four samples. This is noteworthy because *Arcobacter* spp. (which are closely related to *Campylobacter* and *Helicobacter* genera) are considered emergent enteropathogens and potential zoonotic agents [28].

Even though the specificity of the PCRs for Helicobacteraceae (primers C97-C98, and Hel3-Hel4 for the nested PCR) was confirmed *in silico*, one out of the four isolated *Arcobacter* strains (*A. butzleri* IZSLER-260361) cross-reacted with primers C97-C98, while the milk from which it was isolated was negative. Also the 23S rRNA *Helicobacter* gene PCR [18] amplified all the four *Arcobacter* strains isolated in this study, as well as *Campylobacter jejuni* ATCC 49943 (data not shown). According to these findings, these primers targeting 16S and 23S rRNA genes of *Helicobacter* spp. can react unspecifically with non-Helicobacteraceae species. Thus the detection of Helicobacteraceae from the three bulk tank milk samples of our study should be considered questionable, raising concerns also about the results obtained from milk with the cited 16S rRNA gene targeting PCR reported in the literature [29]. Further studies based on a metagenomic approach will be able to confirm or exclude the presence of Helicobacteraceae in these positive bulk tank milk samples, providing also an identification at species level.

4. Conclusions

The results of this study revealed that *H. pylori* is not present in the bulk tank milk and indicated a negligible prevalence of Helicobacteraceae in raw milk of dairy cattle, suggesting that milk is not a transmission vehicle of this infection, at least in the examined geographic area and under the investigated farming conditions.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

The Prevalence of *Helicobacter pylori* Virulence Factors in Bhutan, Vietnam, and Myanmar Is Related to Gastric Cancer Incidence

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Gastric cancer is a significant health problem in Asia. Although the prevalence of *Helicobacter pylori* infection is similar in Bhutan, Vietnam, and Myanmar, the incidence of gastric cancer is highest in Bhutan, followed by Vietnam and Myanmar. We hypothesized that *H. pylori* virulence factors contribute to the differences. The status of *cagA*, *vacA*, *jhp0562*, and *β-(1,3)galT(jhp0563)* was examined in 371 *H. pylori*-infected patients from Bhutan, Vietnam, and Myanmar. Each virulence factor could not explain the difference of the incidence of gastric cancer. However, the prevalence of quadruple-positive for *cagA*, *vacA* s1, *vacA* m1, and *jhp0562*-positive/*β-(1,3)galT*-negative was significantly higher in Bhutan than in Vietnam and Myanmar and correlated with gastric cancer incidence. Moreover, gastritis-staging scores measured by histology of gastric mucosa were significantly higher in quadruple-positive strains. We suggest that the *cagA*, *vacA* s1, *vacA* m1, and *jhp0562*-positive/*β-(1,3)galT*-negative genotype may play a role in the development of gastric cancer.

1. Introduction

Many studies indicate that the highest incidence rate of gastric cancer (GC) is found in East Asia (highest in Korea, followed by Mongolia, Japan, and China) [1]. However, the incidence of GC in South Central and South East Asia is variable and still unclear. The age standardized incidence rate (ASR) of GC is reported to be high in some countries such as Kazakhstan or Bhutan and to a lesser extent Vietnam, whilst it is lower in Myanmar and India (accessible at

http://globocan.iarc.fr/). Bhutan, a small landlocked country in South Asia, has no national guidelines or recommendations for GC screening. Data from GLOBOCAN 2008 showed the ASR of GC in Bhutan (24.2 cases/100,000 per year) to be higher than that in Kazakhstan (20.6/100,000 per year), Vietnam (18.9/100,000 per year, approximately 1.28 times lower than in Bhutan), or Myanmar (11.0/100,000 per year, approximately 2.2 times lower than in Bhutan). However, four years later, data from GLOBOCAN 2012 reported that the ASR of GC in Bhutan had fallen to 17.2 cases/100,000 per year. This was still higher than the ASR of GC in Vietnam and Myanmar, but lower than that of Kazakhstan (21.6/100,000 per year). However, estimates of ASR in Bhutan are somewhat uncertain, because of the extremely low number of GC cases reported (e.g., only 92 cases in GLOBOCAN 2012). Indeed, when we performed a survey using gastroduodenal endoscopy in Bhutan in 2010, we found five cases of GC among 372 volunteers [2]. In our second survey performed in 2014, we also found six cases of GC among 470 volunteers (unpublished data). Therefore, we believe that the actual number of GC patients in Bhutan is higher than previously estimated.

The association between *H. pylori* infection and GC is well established [3, 4], but high prevalence of *H. pylori* infection is not always associated with high incidence of GC. For example, despite the high *H. pylori* infection rate in India, the incidence of GC is low, a phenomenon that has been termed the "Asian enigma" [5]. Moreover, many studies have demonstrated that virulence factors of *H. pylori* such as *cagA* and *vacA* play important roles in the severe *H. pylori* infection-mediated gastric diseases and contribute partly to the geographic variation in the ASR of GC [6–9]. Additionally, a number of recent studies indicated that the novel *H. pylori* factors *jhp0562* and *β-(1,3)galT* are associated with peptic ulcer diseases [10, 11]. *Jhp0562* encodes a glycosyltransferase involved in the synthesis of lipopolysaccharide (LPS); *β-(1,3)galT* shares a high level of sequence similarity with *jhp0562* and is involved in the Lewis (Le) antigen expression of LPS. The presence of *jhp0562* alone (*jhp0562*-positive/*β-(1,3)galT*-negative) is associated with peptic ulcers rather than with gastritis. Our previous study indicated that the prevalence of *jhp0562*-positive/*β-(1,3)galT*-negative genotype was very high among Japanese strains and low among the US strains [10]. Therefore, together with virulence factors such as *cagA* and *vacA*, *jhp0562* and *β-(1,3)galT* might be predictors of severe clinical outcomes from *H. pylori* infection, as well as of GC.

Because the differences in GC incidence can be explained in part by differences between *H. pylori* strains [9], we aimed to examine the prevalence of *H. pylori* virulence factors (*cagA*, *vacA, jhp0562*, and *β-(1,3)galT*) in three South Asian countries with different incidences of GC: Bhutan, Vietnam, and Myanmar.

2. Methods

2.1. Patients. *H. pylori* strains were obtained from the gastric mucosa of *H. pylori*-infected subjects in Bhutan, Vietnam, and Myanmar. *H. pylori* strains were obtained in three cities (Thimphu, Punakha, and Wangdue) in Bhutan in 2010 [2, 12] and two cities (Yangon and Mandalay) in Myanmar in 2012 [13]. In Vietnam, we used samples isolated in two cities (Hanoi and Ho Chi Minh) in 2008 [7]. All the subjects from these countries were selected from our previous studies [7, 12, 13]. In each city, an endoscopy survey was performed over a continuous period of three to five days and all volunteers meeting the inclusion criteria were enrolled in this study. We included subjects greater than 16 years old with dyspeptic symptoms. Subjects with a history of partial gastric resection were excluded. Subjects who received *H. pylori* eradication therapy or treatment with antibiotics, bismuth-containing compounds, H_2-receptor blockers, or proton pump inhibitors within four weeks prior to the study were also excluded.

Presentation included gastritis, duodenal ulcer (DU), gastric ulcer (GU), and GC. DU, GU, and GC were identified by endoscopy. Written informed consent was obtained from all participants, and the protocol was approved by the Ethics Committees of Jigme Dorji Wangchuck National Referral Hospital in Bhutan, Cho Ray Hospital and Bach Mai Hospital in Vietnam, Yangon General Hospital and Mandalay General Hospital in Myanmar, Thammasat University Hospital in Thailand, and Oita University Faculty of Medicine in Japan.

2.2. H. pylori Genotyping. Antral biopsy specimens were obtained for the isolation of *H. pylori* using standard culture methods as previously described [14]. *H. pylori* DNA was extracted using the QIAamp DNA Mini Kit (QIAGEN, Valencia, CA) and used to analyze *H. pylori* genotyping. The status of *cagA, vacA* s1, *vacA* m1, *jhp0562*, and *β-(1,3)galT* was determined by polymerase chain reaction (PCR) as described previously [15–18]. The primer sets used are described in Table 1. PCR amplification for *jhp0562* and *β-(1,3)galT* was performed using one primer pair, 5′-TGA AAA GCC CTT TTG ATT TTG-3′ and 5′-GCT GTA GTG GCC ACA TAC ACG-3′, as described previously [18]. *H. pylori* strain 26695 (ATCC 700392), which is negative for *jhp0562* and positive for *β-(1,3)galT*, and strain J99 (ATCC 700824), which is positive for both genes, were used as the reference strains [10]. The primers generated two PCR products with 301 and 602 bp in strain J99, corresponding to *jhp0562* and *β-(1,3)galT*, respectively, and only one PCR product with 558 bp in strain 26695, corresponding to *β-(1,3)galT*(*hp0619*). The amplified fragments were separated and visualized by gel electrophoresis.

2.3. Staging for Gastritis. All biopsy materials were fixed in 10% buffered formalin for 24 h and then embedded in paraffin. Serial sections were stained with hematoxylin and eosin. Histological analysis of the gastric mucosa was performed according to the Updated Sydney System [19]. The degree of inflammation, neutrophil activity, atrophy, intestinal metaplasia, and bacterial density was classified into four grades: 0, normal; 1, mild; 2, moderate; and 3, marked. In addition, on the basis of the topographic locations (antrum and corpus), the gastritis stage (the severity and topography

Table 1: Primer sequences.

Genes	Primer sequences ($5' \rightarrow 3'$)	PCR product (bp)	PCR conditions
cagA	ACC CTA GTC GGT AAT GGG GCT TTA GCT TCT GAY ACY GC*	521	94°C, 1 min; 52°C, 1 min; 72°C, 1 min (35 cycles)
vacA			
s1/s2	ATG GAA ATA CAA CAA ACA CAC CTG CTT GAA TGC GCC AAA C	259/268	94°C, 1 min; 52°C, 1 min; 72°C, 1 min (35 cycles)
m1/m2	CAA TCT GTC CAA TCA AGC GAG GCG TCA AAA TAA TTC CAA GG	567/642	
jhp0562/*β-(1,3)galT*	TGA AAA GCC CTT TTG ATT TTG GCT GTA GTG GCC ACA TAC ACG	301/602	95°C, 30 s; 56°C, 30 s; 72°C, 1 s (35 cycles)

*Y = C + T.

of atrophy) was assessed according to the Operative Link on Gastritis Assessment (OLGA) system [20, 21].

2.4. Statistical Analysis. Variables such as gender, mean age, and the status of *cagA*, *vacA*, *jhp0562*, and *β-(1,3)galT* were evaluated. The chi-square test was used to examine the association between each genotype and country or clinical outcomes. A multivariate logistic regression model was used to calculate the odds ratios (OR) of the clinical outcomes by including age, sex, and the *H. pylori* genotypes. All determinants with P values of <0.10 were entered together in the full model of logistic regression, and the model was reduced by excluding variables with P values of >0.10. Spearman rank coefficients (r) were also determined to evaluate the association between the different genotypes of the strains. A P value of <0.05 was accepted as statistically significant. SPSS version 19.0 (SPSS, Inc., Chicago, IL) was used for all statistical analyses.

3. Results

3.1. Prevalence of Gastric Diseases and Virulence Factor. A total of 371 *H. pylori* strains were successfully cultured: 200 from Bhutan (161 with gastritis, 18 with DU, 20 with GU, and 1 with GC), 102 from Vietnam (76 with gastritis, 14 with DU, 12 with GU, and no GC cases), and 69 from Myanmar (66 with gastritis, 1 with DU, 1 with GU, and 1 with GC). The characteristics of each study population are described in Table 2. The average age in Vietnam (44.5 ± 13.0) was significantly higher than in Myanmar (40.1 ± 11.5) or Bhutan (36.6 ± 13.8). There was no difference between the male: female ratio of patients from each country. However, in Bhutan, the percentage of male patients was significantly higher in the peptic ulcer group than in the gastritis group (71.1% versus 41.0%, $P = 0.001$). In Vietnam, the percentage of male patients was significantly higher in the peptic ulcer group (65.4% versus 39.5%, $P = 0.02$). In Myanmar, only three peptic ulcer cases were found. The distribution of the status of *cagA*, *vacA*, *jhp0562*, and *β-(1,3)galT* in the three countries is also shown in Table 2.

3.2. Virulence Factors of H. pylori and Clinical Outcomes. First, we examined the association between *H. pylori* genotypes and clinical outcomes (Table 3). In Bhutan, there were no significant differences in the presence/status of *H. pylori* virulence factors between the gastritis and peptic ulcer groups. In Vietnam, the prevalence of *β-(1,3)galT* was significantly higher in strains isolated from patients with peptic ulcer compared with gastritis patients (30.8% versus 13.2%, $P = 0.04$). The presence of *jhp0562* single-positive strains was significantly higher in the gastritis group than in the peptic ulcer group (86.8% versus 69.2%, $P = 0.04$). The prevalence of strains double-positive for *jhp0562* and *β-(1,3)galT* was significantly higher in the peptic ulcer group than in the gastritis group (26.9% versus 10.5%, $P = 0.04$). However, after adjustment for age and gender, these differences did not reach statistical significance ($P = 0.11$ for all cases). In Myanmar, peptic ulcer was found in only three cases; we therefore could not analyze the differences between gastritis and peptic ulcer for this group.

3.3. Gastritis Scores in Patients with Gastritis in Each Studied Country. Next, we included the strains isolated only from subjects with gastritis for histological analyses (Table 4). This group comprised 161 patients from Bhutan, 76 from Vietnam, and 66 from Myanmar. Average age was 44.1 ± 12.7 years old in Vietnam, which was significantly higher than Bhutan and Myanmar (36.8 ± 13.4 and 40.1 ± 11.7, $P < 0.001$ and $P = 0.03$, resp.). There was no difference in the male : female ratio between countries.

Patients from Bhutan showed significantly higher scores for activity, atrophy, and intestinal metaplasia in the antrum than patients from Vietnam ($P < 0.001$, < 0.001, and $P = 0.03$, resp.). Inflammation in the corpus was also significantly higher in Bhutanese than in Vietnamese patients ($P = 0.01$). The atrophic score in the antrum and corpus in subjects from Bhutan was significantly higher than that in Myanmar ($P < 0.001$ and $P = 0.001$, resp.). Vietnamese subjects showed significantly higher atrophic score in the corpus than that of Myanmar ($P < 0.001$), whereas the score of activity in the antrum was significantly higher in Myanmar than Vietnam ($P = 0.02$).

Table 2: Characteristics of *Helicobacter pylori*-infected patients in Bhutan, Vietnam, and Myanmar.

	Bhutan		Vietnam		Myanmar	
n	200		102		69	
Mean age	36.6 ± 13.8*†		44.5 ± 13.0		40.1 ± 11.5**	
Male	94	(47.0%)	47	(46.1%)	28	(40.6%)
Gastric cancer	1	(0.5%)	0	(0.0%)	1	(1.4%)
Peptic ulcer	38	(19.0%)	26	(25.5%)	2	(2.9%)
Gastritis	161	(80.5%)	76	(74.5%)	66	(95.7%)
cagA	200	(100.0%)*†	97	(95.1%)	61	(88.4%)**
vacA s1	200	(100.0%)	102	(100.0%)	67	(97.1%)
vacA m1	154	(77.0%)*†	48	(47.1%)	61	(88.4%)**
jhp0562-positive	197	(98.5%)	99	(97.1%)	67	(97.1%)
β-(1,3)galT-positive	34	(17.0%)†	18	(17.6%)	50	(72.5%)**
jhp0562-positive/*β-(1,3)galT*-negative	166	(83.0%)†	84	(82.4%)	19	(27.5%)**
jhp0562-negative/*β-(1,3)galT*-positive	3	(1.5%)	3	(2.9%)	2	(2.9%)
Double-positive of *jhp0562* and *β-(1,3)galT*	31	(15.5%)†	15	(14.7%)	48	(69.6%)**
cagA/*vacA* s1m1	154	(77.0%)*	46	(45.1%)	55	(79.7%)**
cagA/*vacA* s1m1/*jhp0562*-positive/*β-(1,3)galT*-negative	128	(64.0%)*†	37	(36.3%)	15	(21.7%)**

∗ indicates a statistically significant difference between Bhutan and Vietnam.
∗∗ indicates a statistically significant difference between Myanmar and Vietnam.
† indicates a statistically significant difference between Bhutan and Myanmar.

Gastritis-staging scores were also classified according to the OLGA staging system. The OLGA score was significantly higher in Bhutan than Vietnam and Myanmar ($P < 0.001$ in both comparisons). There was no difference in OLGA score between Vietnam and Myanmar.

3.4. Virulence Factors of H. pylori in Patients with Gastritis in Each Studied Country. In patients with gastritis, the prevalence of *cagA*-positive strains was significantly higher in Bhutan than Vietnam and Myanmar (100% versus 94.7% and 89.4%, $P = 0.01$ and $P < 0.001$, resp.). All strains in Bhutan and Vietnam had the *vacA* s1 genotype; in Myanmar, all except for two strains had the *vacA* s1 genotype. *vacA* m1 strains were found in 75.8% of gastritis patients in Bhutan, 42.1% in Vietnam, and 87.9% in Myanmar. The prevalence of the *vacA* m1 genotype was significantly higher in Bhutan and Myanmar than Vietnam ($P < 0.001$ in both cases). The prevalence of the *vacA* m1 genotype was significantly higher in strains from Myanmar than those from Bhutan ($P = 0.04$). The prevalence of the *cagA*-positive/*vacA* s1m1 genotype was significantly higher in strains from Bhutan and Myanmar than those from Vietnam (75.8% and 80.3% versus 39.5%, $P < 0.001$ and $P < 0.001$, resp.).

jhp0562-positive strains were found in 98.1% of gastritis patients in Bhutan, 97.4% in Vietnam, and 97.0% in Myanmar. There was no difference in the *jhp0562*-positive rate among the three countries, but the prevalence of *β-(1,3)galT*-positive strains was significantly higher in Myanmar (71.2%) than in Bhutan (18.0%) or Vietnam (13.2%) ($P < 0.001$ in both cases). The prevalence of strains double-positive for *jhp0562* and *β-(1,3)galT* was also significantly higher in Myanmar (68.2%) than in Bhutan (16.1%) or Vietnam (10.5%) ($P < 0.001$ in both cases). There were no significant differences in the prevalence of the *jhp0562*-negative/*β-(1,3)galT*-positive genotype between the three countries (1.9% in Bhutan, 2.6% in Vietnam, and 3.0% in Myanmar). The prevalence of strains quadruple-positive for *cagA*, *vacA* s1, *vacA* m1, and *jhp0562*-positive/*β-(1,3)galT*-negative was 62.1% in Bhutan, which was significantly higher than Vietnam (34.2%) and Myanmar (22.7%) ($P < 0.001$ in both cases). Furthermore, the presence of quadruple-positive status was significantly correlated with OLGA score ($P < 0.0001$). The OLGA score (mean [median]) was significantly higher in quadruple-positive than other types of strains (1.41 [1] versus 1.12 [1], $P < 0.0001$).

3.5. Correlations between cagA, jhp0562, and β-(1,3)galT. In Bhutan, all cases were *cagA*-positive. In Vietnam, a positive correlation between the presence of *cagA* and *jhp0562* was observed ($r = 0.498$, $P < 0.001$). On the other hand, there was no correlation between the presence of *cagA* and *β-(1,3)galT* in Vietnam ($P = 0.18$). In Myanmar, there were also no

Table 3: Patient characteristics and prevalence of *Helicobacter pylori* virulence factors according to clinical outcome.

(a)

Bhutan	Gastritis		Peptic ulcer	
n	161		38	
Mean age	36.8 ± 13.4		35.3 ± 15.3	
Male	66	(41.0%)	27	(71.1%)*
cagA	161	(100.0%)	38	(100.0%)
vacA s1	161	(100.0%)	38	(100.0%)
vacA m1	122	(75.8%)	31	(81.6%)
jhp0562-positive	158	(98.1%)	38	(100.0%)
β-(1,3)gal- positive	29	(18.0%)	4	(10.5%)
jhp0562-positive/*β-(1,3)galT*-negative	132	(82.0%)	34	(89.5%)
jhp0562-negative/*β-(1,3)galT*-positive	3	(1.9%)	0	(0.0%)
Double-positive of *jhp0562* and *β-(1,3)galT*	26	(16.1%)	4	(10.5%)
cagA/*vacA* s1m1	122	(75.8%)	31	(81.6%)
cagA/*vacA* s1m1/*jhp0562*-positive/*β-(1,3)galT*-negative	100	(62.1%)	28	(73.7%)

∗ indicates $P < 0.05$.

(b)

Vietnam	Gastritis		Peptic ulcer	
n	76		26	
Mean age	44.1 ± 12.7		46.0 ± 14.1	
Male	30	(39.5%)	17	(65.4%)*
cagA	72	(94.7%)	25	(96.2%)
vacA s1	76	(100.0%)	26	(100.0%)
vacA m1	32	(42.1%)	16	(61.5%)
jhp0562-positive	74	(97.4%)	25	(96.2%)
β-(1,3)galT-positive	10	(13.2%)	8	(30.8%)*
jhp0562-positive/*β-(1,3)galT*-negative	66	(86.8%)	18	(69.2%)*
jhp0562-negative/*β-(1,3)galT*-positive	2	(2.6%)	1	(3.8%)
Double-positive of *jhp0562* and *β-(1,3)galT*	8	(10.5%)	7	(26.9%)*
cagA/*vacA* s1m1	30	(39.5%)	16	(61.5%)
cagA/*vacA* s1m1/*jhp0562*-positive/*β-(1,3)galT*-negative	26	(34.2%)	11	(42.3%)

∗ indicates $P < 0.05$.

correlations between *cagA*, *jhp0562*, and *β-(1,3)galT* ($P = 0.08$ and $P = 0.50$, resp.).

4. Discussion

Jhp0562 has been reported to be associated with peptic ulcer diseases in children, but not in adults, in the Portuguese population [18]. In a subsequent study in Portuguese children by the same group, the presence of *jhp0562* alone (*jhp0562*-positive/*β-(1,3)galT*-negative) was associated with peptic ulcers, whereas the presence of *β-(1,3)galT* alone (*jhp0562*-negative/*β-(1,3)galT*-positive) was associated with gastritis [11]. We previously reported that the prevalence of *jhp0562* was higher in Japan than in the USA (100% versus 78%) [10]. Furthermore, we reported that the *jhp0562*-positive/*β-(1,3)galT*-negative genotype was significantly associated with peptic ulcer in the USA [10]. In the present study, we found that almost all strains isolated in Bhutan, Vietnam, and Myanmar were positive for jhp0562 (98.5%, 97.1%, and 97.2%, resp.). *H. pylori* strains carrying *cagA* and the potentially toxigenic *vacA* s1 alleles were found to predominate in all studied countries, but strains carrying the *vacA* m1 were higher in Bhutan and Myanmar than in Vietnam. We did not identify any relationship between the prevalence of any studied virulence factors and the difference of GC incidences in Bhutan, Myanmar, and Vietnam. It might be similar to the other East Asian countries, where *cagA*-positive were found in almost all strains and *vacA* s1m1 is dominant; therefore, these virulence factors could not show the usefulness as markers of GC. Interestingly, we found the contribution of novel factors *jhp0562* and *β-(1,3)galT* in describing the different incidences of GC between Bhutan, Vietnam, and Myanmar. The prevalence of strains quadruple-positive for *cagA*, *vacA* s1, *vacA* m1, and *jhp0562*-positive/*β-(1,3)galT*-negative was the highest in Bhutan, followed by Vietnam and Myanmar, which correlated with the incidence of GC reported in GLOBOCAN 2012 (http://globocan.iarc.fr/). Furthermore, the presence of the quadruple-positive genotype

Table 4: *Helicobacter pylori* virulence factors and gastritis scores in patients with gastritis in three countries.

	Bhutan		Vietnam		Myanmar	
n	161		76		66	
Mean age	36.8 ± 13.4*†		44.1 ± 12.7		40.1 ± 11.7**	
Male	66	(41.0%)	30	(39.5%)	27	(40.9%)
cagA	161	(100.0%)*†	72	(94.7%)	59	(89.4%)
vacA s1	161	(100.0%)	76	(100.0%)	64	(97.0%)
vacA m1	122	(75.8%)*†	32	(42.1%)	58	(87.9%)**
jhp0562-positive	158	(98.1%)	74	(97.4%)	64	(97.0%)
β-(1,3)galT-positive	29	(18.0%)†	10	(13.2%)	47	(71.2%)**
jhp0562-positive/*β-(1,3)galT*-negative	132	(82.0%)†	66	(86.8%)	19	(28.8%)**
jhp0562-negative/*β-(1,3)galT*-positive	3	(1.9%)	2	(2.6%)	2	(3.0%)
Double-positive of *jhp0562* and *β-(1,3)galT*	26	(16.1%)†	8	(10.5%)	45	(68.2%)**
cagA/*vacA* s1m1	122	(75.8%)*	30	(39.5%)	53	(80.3%)**
cagA/*vacA* s1m1/*jhp0562*-positive/*β-(1,3)galT*-negative	100	(62.1%)*†	26	(34.2%)	15	(22.7%)
Antrum						
Activity	1.53 (1)*		1.20 (1)		1.44 (1)**	
Inflammation	1.76 (2)		1.67 (2)		1.65 (2)	
Atrophy	1.42 (1)*†		0.89 (1)		0.89 (1)	
Intestinal metaplasia	0.16 (0)*		0.04 (0)		0.14 (0)	
Corpus						
Activity	0.93 (1)		0.92 (1)		0.88 (1)	
Inflammation	1.14 (1)*		1.23 (1)		1.08 (1)**	
Atrophy	0.55 (0)†		0.58 (1)		0.26 (0)**	
Intestinal metaplasia	0.02 (0)		0.05 (0)		0.00 (0)	
OLGA	1.51 (1)*†		1.01 (1)		0.94 (1)	

∗ indicates a statistically significant difference between Bhutan and Vietnam.
∗∗ indicates a statistically significant difference between Myanmar and Vietnam.
† indicates a statistically significant difference between Bhutan and Myanmar.
For histological scores (minimum 0 to maximum 3) and OLGA score (minimum 0 to maximum 4), mean (median) is presented.

was significantly correlated with the OLGA score. These results suggest that the prevalence of strains quadruple-positive for *cagA*, *vacA* s1, *vacA* m1, and *jhp0562*-positive/*β-(1,3)galT*-negative might be a marker for the development of GC. Our finding proved the essential element of virulence factors over *H. pylori* infection in evaluating the higher incidence of GC between the countries with the same *H. pylori* prevalence. The limitation of the study is that only very few strains were obtained from GC; therefore, the true prevalence of these quadruple-positive factors in GC is difficult to estimate.

In this study, there were no differences in the prevalence of *H. pylori* virulence factors including *cagA*, *vacA*, *jhp0562*, and *β-(1,3)galT* between the gastritis and peptic ulcer groups from Bhutan. However, the prevalence of the *cagA*-positive, *vacA* s1m1, and *jhp0562*-positive/*β-(1,3)galT*-negative genotype was higher in Bhutan than in Vietnam or Myanmar. In addition, all *cagA*-positive strains in Bhutan were also positive for *jhp0562*. The prevalence of *jhp0562* was significantly positively correlated with *cagA* in Vietnam. In our previous study, the prevalence of *jhp0562* in the *cagA*-positive strains was 100% in Japan, and the prevalence of *jhp0562* was strongly associated with that of *cagA* in the US population [10]. *cagA* status is also linked to the *vacA* s region type, and it is further closely linked to the presence of *babA* and *oipA* "on" status, which are virulence factors coding for outer membrane proteins [22–24]. As a result, almost all *H. pylori* strains circulating in Japan are extremely virulent, harboring the *cagA*, *vacA* s1 genotype, *oipA* "on" status, and *babA* irrespective of clinical outcomes [24–26]. Therefore, *H. pylori* strains isolated from Bhutan can also be considered highly virulent. Thus, we suggested that the phenotype resulting from the expression of *cagA*, *vacA* s1m1, and *jhp0562* confers a biological advantage to the strains, with the cumulative action of each factor contributing simultaneously to the fitness of the strains *in vivo* and a more pronounced proinflammatory response. It might be better to hypothesize that these factors interact synergistically with each other and induce serious diseases than to discuss which of these factors is the most virulent. Our suggestion might partly explain the correlation between the higher prevalence of strains with quadruple-positive virulence and increased GC incidence.

It remains unclear whether the presence of the *jhp0562*-positive/*β-(1,3)galT*-negative genotype is associated with higher gastritis score. *jhp0562* encodes a glycosyltransferase involved in the synthesis of the chemical structure of LPS. *H. pylori* expresses Le antigens in its LPS, both type 1 (Le^a and Le^b) and type 2 (Le^x and Le^y); these are structurally related

to the human blood group antigens also expressed in gastric epithelial cells [27, 28]. *jhp0562* is located immediately upstream of *β-(1,3)galT*, which in turn encodes a *β-(1,3)* galactosyltransferase involved in type I Le antigen synthesis [29, 30]. The *jhp0562* and *β-(1,3)galT* genes are highly similar (>80%), especially at their 5′ and 3′ ends [31]. Although these Le antigenic structures were reported to be important for bacterial colonization, adhesion, and evasion of host immune response [32–34], the role of these in *H. pylori* infection has not been elucidated. A recent study reported that mutagenesis of *jhp0562* resulted in the loss of expression of all Le types, suggesting that the product of this gene is truly an essential glycosyltransferase for Le expression [31]. These successive complementation results showed that *β-(1,3)galT* alone was insufficient for type 1 Le synthesis and that *jhp0562* must also be present. *jhp0562* contributed to both type 1 and type 2 Le synthesis, while *β-(1,3)galT* was essential only for type 1 Le synthesis. Another report revealed that East Asian strains express types 1 and 2 Le antigens, whereas Western strains express only type 2 Le antigens [35]. It has also been shown that the expression of Le^x or Le^y in *H. pylori* isolates is significantly higher in peptic ulcer than in nonulcer dyspepsia patients [36]. On the other hand, another study in China, where most strains express Le^x or Le^y, did not confirm this relationship [37]. This is similar to *cagA*, which cannot be used as a marker in areas where the incidence of GC is high. *In vitro* and *in vivo* studies are necessary to elucidate the causal relationship between *jhp0562* and *β-(1,3)galT* and to investigate the mechanisms by which these gene products correlate with clinical outcomes.

5. Conclusion

In conclusion, each virulence factor including *cagA*, *vacA* s1, m1, *jhp0562,* and *β-(1,3)galT* could not explain the difference of the incidence of GC between Bhutan, Vietnam, and Myanmar. Interestingly, the prevalence of the quadruple-positive genotype for *cagA*, *vacA* s1, *vacA* m1, and *jhp0562*-positive/*β-(1,3)galT*-negative was highest in Bhutan, followed by Vietnam and Myanmar, which correlated with the incidence of GC. Furthermore, the presence of the quadruple-positive genotype was significantly correlated with severe gastritis. This suggests that the prevalence of the quadruple-positive genotype for *cagA*, *vacA* s1, *vacA* m1, and *jhp0562*-positive/*β-(1,3)galT*-negative might be a marker for the development of GC.

Abbreviations

ASR: Age standardized incidence rate
DU: Duodenal ulcer
GC: Gastric cancer
LPS: Lipopolysaccharide
PCR: Polymerase chain reaction.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Clinical Study

Comparison of Second-Line Quadruple Therapies with or without Bismuth for *Helicobacter pylori* Infection

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The bismuth-based quadruple regimen has been applied in *Helicobacter pylori* rescue therapy worldwide. The non-bismuth-based quadruple therapy or "concomitant therapy" is an alternative option in first-line eradication but has not been used in second-line therapy. Discovering a valid regimen for rescue therapy in bismuth-unavailable countries is important. We conducted a randomized controlled trial to compare the efficacies of the standard quadruple therapy and a modified concomitant regimen. One hundred and twenty-four patients were randomly assigned into two groups: RBTM (rabeprozole 20 mg bid., bismuth subcitrate 120 mg qid, tetracycline 500 mg qid, and metronidazole 250 mg qid) and RATM (rabeprozole 20 mg bid., amoxicillin 1 g bid., tetracycline 500 mg qid, and metronidazole 250 mg qid) for 10 days. The eradication rate of the RBTM and RATM regimen was 92.1% and 90.2%, respectively, in intention-to-treat analysis. Patients in both groups had good compliance (~96%). The overall incidence of adverse events was higher in the RATM group (42.6% versus 22.2%, $P = 0.02$), but only seven patients (11.5%) experienced grades 2-3 events. In conclusion, both regimens had good efficacy, compliance, and acceptable side effects. The 10-day RATM treatment could be an alternative rescue therapy in bismuth-unavailable countries.

1. Introduction

Helicobacter pylori (H. pylori) causes several gastrointestinal diseases including peptic ulcers, gastric adenocarcinoma, and mucosa associated lymphoid tissue lymphoma (MALToma); eradication of *H. pylori* is recommended in these conditions [1]. The standard 7-day triple therapy including a proton pump inhibitor (PPI), amoxicillin, and clarithromycin is

the first-line treatment for *H. pylori*. However, its failure rate has increased to almost 20% in Taiwan [2, 3] and around 60% of countries worldwide fail to reach an eradication rate of more than 80% [4–6]. The standard quadruple therapy consisting of PPI, bismuth salt, tetracycline, and metronidazole is widely used as the first-line treatment if clarithromycin resistance rate is more than 20%. The 3rd Brazilian consensus, 2013, and Maastricht IV consensus [7, 8] also recommended it as a second-line salvage therapy. However, bismuth is not available in many countries; thus, an equally effective non-bismuth-based quadruple therapy is essential for *H. pylori* treatment [9].

The non-bismuth-based quadruple therapy, consisting of the standard triple therapy (PPI, amoxicillin, and clarithromycin) plus either metronidazole or tinidazole, is also known as "concomitant therapy" [9]. It has been used as an alternative first-line eradication regimen [10, 11]. However, clarithromycin has been included in the first-line triple therapy and the secondary *H. pylori* resistance rates in Taiwan are higher in clarithromycin (29.7–45.7%) and metronidazole (40–58.7%) and lower in amoxicillin (4.3~6%) and tetracycline (0%) [3, 12, 13]. Therefore, we modified the standard concomitant therapy by omitting clarithromycin and designed a randomized study to compare the performance of two rescue regimens: RBTM (rabeprazole, bismuth subcitrate, tetracycline, and metronidazole) and RATM (rabeprazole, amoxicillin, tetracycline, and metronidazole). To the best of our knowledge, it is the first study to directly compare the two regimens as the second-line therapy.

2. Material and Methods

2.1. Study Population, Therapy Protocols, and Confirmation of H. pylori Status. All patients who had persistent *H. pylori* infection after the standard first-line triple therapy (PPI bid., clarithromycin 500 mg bid., and amoxicillin 1 g bid. for 7 days) were enrolled from two medical centers, Kaohsiung Medical University Hospital and Kaohsiung Veterans General Hospital in Kaohsiung, Taiwan, between November 2009 and October 2011. The rapid urease test, histology, and culture were not performed in all patients. Some patients only received ^{13}C urea breath test to confirm the presence of *H. pylori*. Hence the definition of "the presence of *H. pylori*" was (1) positive results of both rapid urease test and histology, (2) positive culture result, or (3) positive finding of ^{13}C urea breath test. The exam of rapid urease test, histology, and culture was performed in 79 patients. The results of culture revealed 35 positive findings and 44 negative findings. In the 44 patients with negative finding of *H. pylori* culture was confirmed by positive results of both rapid urease test and histology. The rest of patients in this study only received ^{13}C urea breath test to confirm the presence of *H. pylori*. The exclusion criteria included (a) ingestion of antibiotics, bismuth, or PPI within 4 weeks before our intervention; (b) a history of allergy to the medications used; (c) previous gastric surgery; (d) the coexistence of serious concomitant illness such as decompensated liver cirrhosis and uremia; and (e) pregnant or lactating women.

The participants were randomly assigned into the 10-day treatment groups by using a computer number table. The RBTM regimen consisted of rabeprazole 20 mg bid, bismuth subcitrate 120 mg qid, tetracycline 500 mg qid, and metronidazole 250 mg qid, and the RATM consisted of rabeprazole 20 mg bid, amoxicillin 1 g bid, tetracycline 500 mg qid, and metronidazole 250 mg qid. The participants were asked to return 1-2 weeks after the treatment course for a questionnaire interview and to count the residual tablets. ^{13}C urea breath test was performed to confirm their *H. pylori* status 4 weeks later. All participants gave written informed consent. This study was approved by the Institutional Review Board of Kaohsiung Medical University.

2.2. Questionnaire. The indexes of questions included sex, age, underlying systemic disease, and smoking and alcohol-drinking habits. The details of adverse effects in the questionnaire included diarrhea, constipation, abdominal pain, anorexia, nausea, vomiting, skin rash, headache, dizziness, bad taste, and fatigue, among others. We differentiated the different degrees of adverse effect into four grades including 0: none; 1: feeling discomfort but can take daily activity and work normally; 2: feeling discomfort and affecting their daily activity or work; 3: feeling too much discomfort to take the drug, causing discontinuation of the treatment course. The definition of poor compliance was completing the therapy course of less than 70% [14].

2.3. Statistical Analysis. The *H. pylori* eradication rates were evaluated by intention-to-treat (ITT) and per-protocol (PP) analyses. ITT analysis was defined as comparing all patients enrolled in the two groups. Those who did not return for a ^{13}C urea breath test were deemed as dropout. PP analysis was defined as comparing two groups of patients who completed the whole treatment course and received *H. pylori* follow-up. The characteristics, eradication rates, and presence of adverse events were calculatedly by the Chi-square test. Student's t-test was used to compare the patient's ages in the two groups. A P value less than 0.05 was considered statistically significant and all P values were two-sided. The software of SPSS was used for statistical analysis (IBM Corp. version 19). Assuming that the eradication rate of the RBTM group was 70% [3], and the RATM group achieved a 90% eradication rate [15], a 20% increase, our statistical power in this study is 80% under the sample size of about 60 subjects in each group and the two-sided P value is 0.05 if 95% of patients completed the follow-up.

3. Results

The flow chart of study design and randomization protocol is shown in Figure 1. One hundred and thirty patients were enrolled in this study; six of them were excluded according to exclusion criteria. The remaining 124 patients were randomly assigned into the RBTM ($N = 63$) and RATM ($N = 61$) groups. One patient in the RBTM group and three patients in the RATM group did not return to confirm *H. pylori* status and were deemed dropout in the ITT analysis. Two patients

Table 1: Characteristics of the participants receiving different eradication regimens.

	RBTM group ($n = 63$)	RATM group ($n = 61$)	P value
Age (years)	55.0 ± 12.1	54.1 ± 12.0	0.68
Sex			
Male	24 (38.1%)	33 (54.1%)	0.07
Female	39 (61.9%)	28 (45.9%)	
Smoking	5 (7.9%)	7 (11.5%)	0.51
Alcohol drinking	3 (4.8%)	2 (3.3%)	0.68
Diagnosis			
Gastritis	29 (46.0%)	26 (42.6%)	
Gastric ulcer	7 (11.1%)	7 (11.5%)	
Duodenal ulcer	17 (27.0%)	23 (37.7%)	0.57
Peptic ulcer*	1 (1.6%)	1 (1.6%)	
Others	9 (14.3%)	4 (6.6%)	

*Peptic ulcer: concurrent gastric ulcer and duodenal ulcer.
RBTM: rabeprazole, bismuth subcitrate, tetracycline, and metronidazole.
RATM: rabeprazole, amoxicillin, tetracycline, and metronidazole.

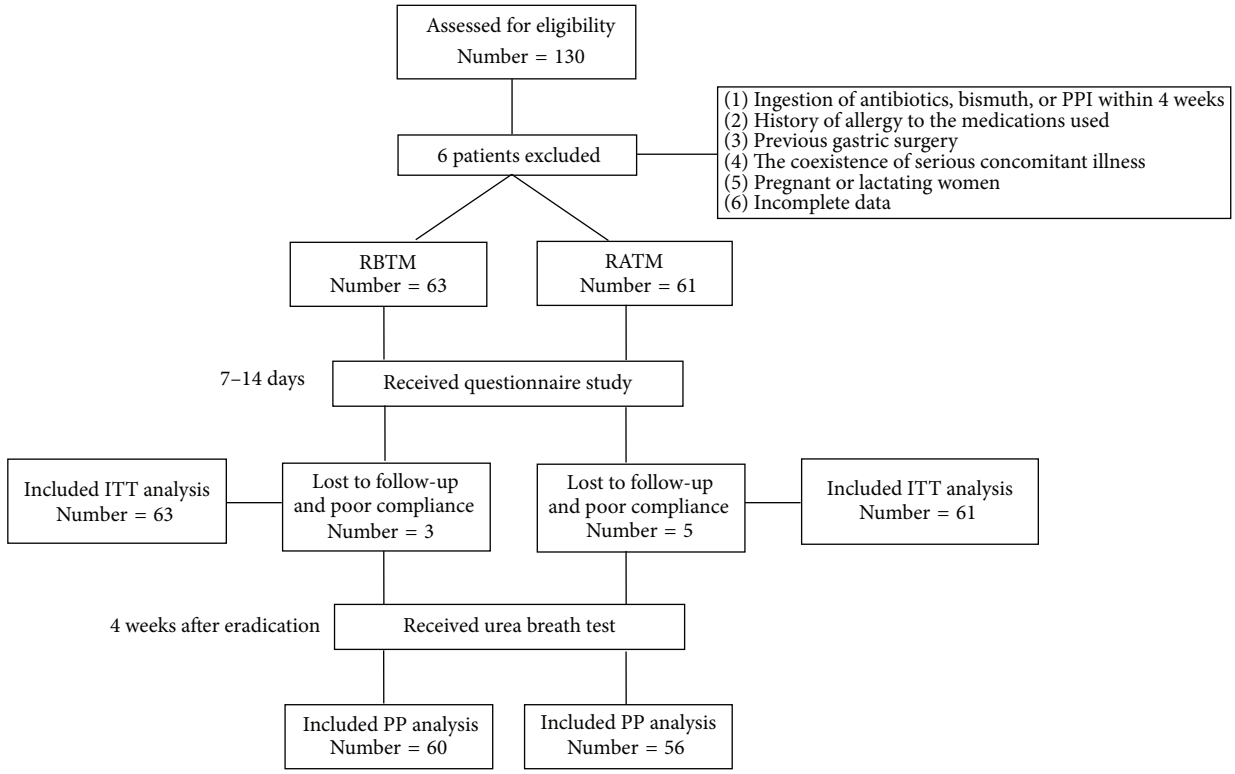

Figure 1: Flow diaphragm of study design through randomization.

in the RBTM group and two patients in the RATM group completed less than 70% of therapy course and were deemed incomplete therapy course in the ITT analysis.

The demographic characteristics of study participants were not significantly different between the two groups. The most common endoscopic diagnosis in our study was gastritis (RBTM: 46.0% versus RATM: 42.6%), followed by duodenal ulcer (RBTM: 27.0% versus RATM: 37.7%) (Table 1). The *H. pylori* eradication rates of the RBTM and RATM regimens were 92.1% versus 90.2% in ITT analysis and 93.3% versus 89.3% in PP analysis. The compliance between the two groups was also similar (RBTM: 96.8% versus RATM: 96.7%, $P = 0.97$). The overall rate of adverse events was 22.2% (14/63) in the RBTM group and 42.6% (26/61) in the RATM group ($P = 0.02$) (Table 2). Although more adverse events were reported in the RATM group, only seven patients had severity more than grades 2 or 3. The rest of the study participants with discomfort experience were only assessed at grade 1. Dizziness (8 versus 0 cases, $P = 0.03$) and headache (7 versus 1 case, $P = 0.08$) were more common in the RATM than in

Table 2: The outcomes of RBTM and RATM treatment regimens.

	RBTM group ($n = 63$)	RATM group ($n = 61$)	P value
Eradication rate			
Intention-to-treat	92.1% (58/63)	90.2% (55/61)	0.71
Per-protocol	93.3% (56/60)	89.3% (50/56)	0.44
Compliance	96.8% (61/63)	96.7% (59/61)	0.97
Adverse events	22.2% (14/63)	42.6% (26/61)	0.02

Table 3: Adverse events of the RBTM and RATM regimens.

Adverse events	RBTM ($n = 63$)	RATM ($n = 61$)	P value
Diarrhea	2 (2/0/0)	6 (5/1/0)	0.28
Constipation	0 (0/0/0)	0 (0/0/0)	—
Abdominal pain	3 (3/0/0)	7 (5/2/0)	0.25
Anorexia	1 (1/0/0)	2 (0/1/1)	0.39
Nausea	5 (5/0/0)	12 (8/2/2)	0.14
Vomiting	6 (6/0/0)	10 (8/1/1)	0.46
Skin rash	1 (1/0/0)	2 (0/2/0)	0.22
Headache	1 (1/0/0)	7 (6/1/0)	0.08
Dizziness	0 (0/0/0)	8 (3/3/2)	0.03
Bad taste	2 (2/0/0)	4 (2/2/0)	0.35
Fatigue	2 (2/0/0)	5 (4/1/0)	0.40
Others	0 (0/0/0)	4 (3/0/1)	0.12

Total number of individual adverse events (number of different degrees of adverse events: 1/2/3).

the RBTM group. The most common adverse event in the RATM group was nausea ($N = 12$) (Table 3).

4. Discussion

The Maastricht IV consensus has suggested that metronidazole should be included in the standard second-line quadruple therapy [7]. The concomitant or non-bismuth-based quadruple therapy has not been used as the second-line treatment yet. By replacing clarithromycin with tetracycline, we directly compared the modified concomitant regimen (RATM) with the bismuth-based quadruple therapy (RBTM) for 10 days. We found a comparable efficacy (RBTM 92.1% versus RATM 90.2% in ITT analysis) and compliance (~96%), but variable adverse effects (RBTM 22.2% versus RATM 42.6%, $P = 0.02$) of the rescue quadruple therapies with or without bismuth. The eradication rates of both 10-day regimens were similar to the 14-day quadruple therapy (ITT: 82.6%, PP: 93.6%) [16]. The costs of both regimens were much cheaper than levofloxacin-containing rescue therapy. Moreover, RATM can be a useful alternative in bismuth-unavailable areas. All participants received the same first-line treatment in the study hospitals. Thus, we had a more homogeneous study population.

Only one study used the same antibiotic combination and compared the concomitant therapy (esomeprazole, amoxicillin, tetracycline, and metronidazole) with bismuth-based quadruple therapy (esomeprazole, bismuth, tetracycline, and metronidazole) in the first-line treatment [17]. The eradication rates were ITT: 74% versus 79% and PP: 80.4% versus 89.7%. Moreover, a meta-analysis of the first-line concomitant therapy consisting of PPI, amoxicillin, and clarithromycin plus either metronidazole or tinidazole revealed an 88% eradication rate in ITT analysis [15]. Many studies have compared different bismuth-based quadruple therapies containing different proton pump inhibitors or antibiotics for a variable treatment duration as the second-line treatment. The recently reported eradication rates were 63.9–85.1% in ITT analysis and 82.6–96.2% in PP analysis [3, 12, 14, 16, 18, 19]. The RBTM regimen in our study seems to have a better result.

H. pylori eradication is influenced by many factors, such as antibiotic resistance, therapy duration, drug compliance, intragastric acidity, and CYP2C19 genetic polymorphism [20]. Regarding antibiotic resistance, the worldwide primary *H. pylori*-resistant rates to clarithromycin, metronidazole, amoxicillin, and tetracycline were 17.2%, 26.7%, 11.2%, and 5.9%, respectively [21]. More specifically, in Asia, the clarithromycin (18.9%) and metronidazole (37.1%) resistance is higher, while tetracycline resistance (2.4%) is lower than average [21]. Our previous studies found that the primary resistant rates were 6.6~13.2% to clarithromycin, 26.7~56% to metronidazole, 0~2% to amoxicillin, and 0.6% to tetracycline [2, 22–24]. Moreover, secondary *H. pylori* resistance was even higher to clarithromycin (29.7~45.7%) and metronidazole (40~58.7%), while being similar to amoxicillin (4.3~6%) and tetracycline (0%) [3, 12, 13]. Dual clarithromycin and metronidazole resistance is an important factor influencing the eradication efficacy. Chi et al. reported a 16–18% dual resistance rate in Taiwan and suggested that second-line

quadruple therapy including tetracycline and amoxicillin could improve the eradication efficacy [13]. However, our previous study found that the efficacy of concomitant therapy was not affected by dual resistance (75.0% versus 92.4%, $P = 0.22$) [23]. Despite the high metronidazole resistance in many areas, Katelaris et al. proposed that metronidazole resistance would not be a major cause of quadruple therapy failure because adding PPI with bismuth triple therapy would overcome the high resistance rate of metronidazole [25]. Kuo et al. indicated that longer metronidazole usage (at least 7 days) in second-line therapy could conquer the metronidazole resistant rate and reach a desirable result (ITT: 79%, PP: 91%) [12]. The suggested dose of metronidazole in rescue quadruple therapy varies from 1000 to 2000 mg daily. We chose 1000 mg daily according to the satisfying efficacy of previous studies and better drug compliance [3, 12, 14, 25]. A Korean trial and the review article have suggested that an extended duration up to 10–14 days was more adequate in rescue therapy [16, 26]. In this study, we used metronidazole 250 mg qid for 10 days and found a good result. One of the limitations of this study is lack of information on antibiotic resistance.

We chose rabeprazole-based regimens in this study to minimize the effect of *CYP2C19* polymorphism on PPI clearance and intragastric acidity [27, 28]. The *CYP2C19* polymorphism leads to three phenotypes: the homozygous extensive metabolizer, heterozygous extensive metabolizer, and poor metabolizer. The poor metabolizer is associated with superior efficacy in curing *H. pylori* because of slower PPI clearance and higher intragastric pH level and, thus, higher intragastric concentrations of antibiotics [28]. Although we did not check CYP2C19 polymorphism in this study, 20% of Asian people have poor metabolizing genotype, which is higher than in Western populations [27]. Therefore, the influence of CYP2C19 polymorphism is not considered high here.

The overall adverse effect was more common in the RATM than in the RBTM group (42.6% versus 22.2%). However, the incidence of grades 2-3 events in our RATM group was only 11.5% (7 patients). The only study using amoxicillin, tetracycline, and metronidazole-containing regimens reported an overall adverse event rate of 10% [17]. The most common adverse event in our RATM group was nausea ($N = 12$), but only four patients had grades 2-3 events. Moreover, there were more adverse events when the treatment course got longer [19, 20]. In our RBTM group, the most common side effect was vomiting (10%, 6 cases), which was similar to other reports [18–20]. Nevertheless, in our previous studies, nausea was the most comment event in bismuth-based quadruple therapy [3, 12, 14]. There was a wide range of adverse event incidences (15–46.4%) using quadruple therapy containing PPI, bismuth, tetracycline, and metronidazole, and metallic taste, nausea, vomiting, and headache were most commonly complained of [3, 12, 14, 18–20]. Our overall adverse event rate (22.2%) in the RBTM group was compatible with other studies. A review and meta-analysis found no serious side effect to bismuth-based *H. pylori* eradication unless the subject had allergy to these drugs [29]. Both RBTM and RATM were safe, well tolerated, and with good compliance in our trial. Only two patients in the RATM group had poor compliance due to skin rash ($n = 1$) and unknown reason ($n = 1$). In the RBTM group, two patients took less than 70% of the medication because of nausea and vomiting. However, three of them (75%) had successful *H. pylori* eradication.

In conclusion, the RATM concomitant therapy as a second-line treatment had similar efficacy but more adverse events than the bismuth-based quadruple therapy. It could be an alternative in bismuth-unavailable areas or where intolerance to bismuth is noted. Further randomized study is needed to investigate the influence of secondary antibiotic resistances on the treatment effects.

Conflict of Interests

All authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Guang-Hong Jheng and I-Chen Wu contributed equally to this study.

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Research Article

Correlation between Gastric Mucosal Morphologic Patterns and Histopathological Severity of *Helicobacter pylori* Associated Gastritis Using Conventional Narrow Band Imaging Gastroscopy

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Background and Aim. Identifying specific gastric mucosal morphologic patterns useful for detecting *Helicobacter pylori* associated gastritis and correlation with histopathological severity. *Methods*. The endoscopists classified the C-NBI gastroscopic findings into 5 gastric mucosal morphologic patterns as follows: type 1: regular arrangement of collecting venules, type 2: cone-shaped gastric pits, type 3: rod-shaped gastric pits with prominent sulci, type 4: ground glass-like morphology, and type 5: dark brown patches with bluish margin and irregular border. Biopsies of all of the cases were then evaluated by 5 pathologists for definitive *Helicobacter pylori* diagnosis. *Result*. Type 1 and type 2 patterns were statistically significant in predicting *Helicobacter pylori* negative status (58/60, $P < 0.01$). Type 3, type 4, and type 5 patterns were statistically significant in predicting *Helicobacter pylori* positive status (132/140, $P < 0.01$). Furthermore, the sensitivity, specificity, and positive and negative predictive values of type 3, 4, or 5 morphologies for predicting *Helicobacter pylori* positive were 94.28%, 96.66%, 98.50%, and 87.87%, respectively, correlated well with inflammation grading according to the Sydney classification ($P < 0.01$). *Conclusion*. Our study suggests that gastric mucosal morphologic patterns in the *Helicobacter pylori* infected gastric mucosa can be reliably identified using C-NBI gastroscopy with good correlation with inflammation grading.

1. Introduction

Narrow band imaging (NBI) is an optical image enhancement technique that enhances the vessels and patterns of the gastric mucosa surface. 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, peptic ulcer disease, and gastric malignancy [1, 2]. European guidelines [3] indicate that at least 2 different tests are necessary to make the diagnosis of *Helicobacter pylori* infection. Although gastroscopic features of *Helicobacter pylori* associated gastritis have been reported in the literature, there is a controversy whether *Helicobacter pylori* associated gastritis can be diagnosed by gastroscopic features alone. Most studies have concluded that it is impossible to diagnose *Helicobacter pylori* related gastritis on the basis of gastroscopic findings alone [4–7]. The narrow band imaging (NBI) system is an endoscopic imaging technique for enhanced visualization of mucosal microscopic structure and capillaries in the superficial mucosal layer. Images are obtained by

using narrow band red, blue, and green filters, which are different from conventional red-green-blue filters [8]. Some recent study from the United States has indicated the usefulness of high resolution narrow band imaging gastroscopy for predicting *Helicobacter pylori* infection and the occurrence of intestinal metaplasia in the stomach [9]. However, in daily clinical practice, the high resolution gastroscopy does not seem to be feasible, because it takes more examination time and needs more training and experience of the endoscopist. If specific gastric mucosal morphologic patterns of *Helicobacter pylori* associated gastritis can be identified using C-NBI gastroscopy, this may be useful for "*site specific biopsy*" of areas with suspected *Helicobacter pylori* infection in daily practice. Up to the present time, there has not been strong evidence regarding specific gastric mucosal morphologic patterns of *Helicobacter pylori* associated gastritis using C-NBI gastroscopy. The aim of this study is to identify specific gastric mucosal morphologic pattern for the detection of *Helicobacter pylori* associated gastritis and to evaluate the possible correlation between these gastroscopic findings and histopathological severity using C-NBI gastroscopy as well as the correlation with inflammation grading according to the Sydney classification [10].

2. Materials and Method

2.1. Patients. A total of 200 patients who underwent gastroscopy for the investigation of dyspeptic symptoms were enrolled in the study from January 2014 to November 2014 at the Endoscopic Unit, Department of Surgery, Suranaree University of Technology Hospital (SUTH), Institute of Medicine, Suranaree University of Technology, Nakhon Ratchasima, Thailand. The following exclusion criteria were applied: age below 18 or above 70 years, *Helicobacter pylori* eradication treatment in the previous 2 months, gastric ulcer or duodenal ulcer, suspected or confirmed malignancy on endoscopy, significant medical illnesses and history of previous gastric surgery, and the use of antimicrobials or gastrointestinal medications like PPIs, H2 blockers, or bismuth compounds within the previous 2 months. All patients provided informed consent, and the study was approved by the Institutional Review Board of Suranaree University of Technology, Nakhon Ratchasima, Thailand. The study was performed in accordance with good clinical practice and the guidelines of the Declaration of Helsinki. All patients provided a 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).

2.2. Diagnosis of Helicobacter pylori Infection. A diagnosis of *Helicobacter pylori* infection was made if *Helicobacter pylori* bacteria were seen on both histopathological examinations and the rapid urease test was positive. A recent study from India attempted to define the gold standard of diagnostic tests to determine *Helicobacter pylori* infection status by breaking down the respective sensitivities and specificities. Both sensitivity and specificity of nested PCR have been reported to be 100%. In contrast, the sensitivity and specificity of serological,

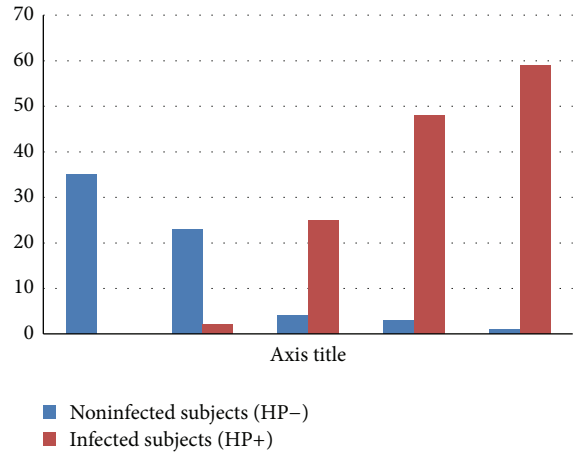


Figure 1: Correlation between gastric mucosal morphologic patterns and *Helicobacter pylori* infection status.

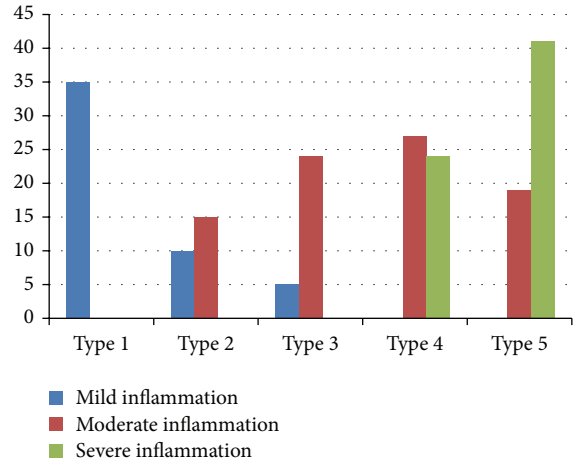
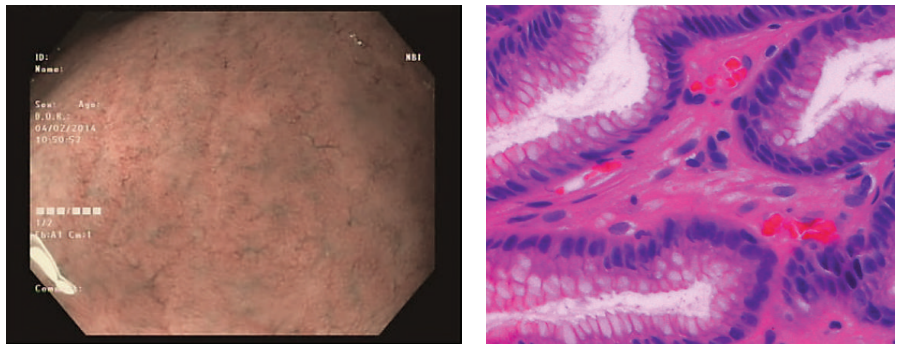


Figure 2: Correlation between gastric mucosal morphologic patterns and inflammation grading.

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 [10]. PCR does not seem to be feasible in daily clinical practice; thus we chose in our study patients to consider them *Helicobacter pylori* negative if they had negative results in one or both of the above selected tests.

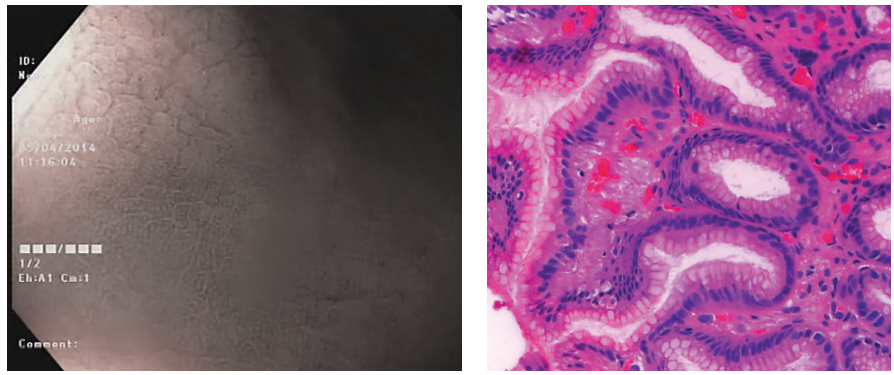
2.3. Biopsy Specimens. Four biopsy samples were taken directly from the observation sites as shown in Figures 3–7. Two samples were sent for histological analysis and 2 were used for rapid urease testing on site (Prontodyle, GASTREX, France).

2.4. Histological Analysis. Specimens for histological analysis were placed in 10% formalin solution and routinely processed. The hematoxylin and eosin stain and Giemsa stain were used for identification of *Helicobacter pylori*. All of the



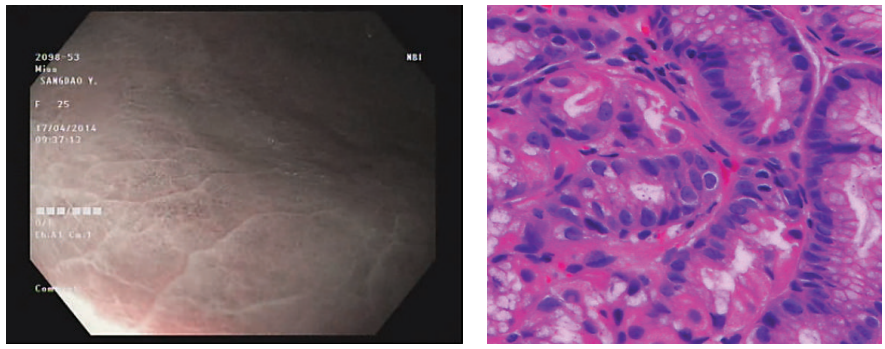
(a) (b)

Figure 3: Type 1 regular arrangement of collecting venules (a). This pattern is associated with regular arrangement of surface epithelium, with absent infiltration by inflammatory cells (b).



(a) (b)

Figure 4: Type 2 cone-shaped gastric pits (a); abnormal C-NBI gastric mucosal morphologic patterns corresponded to mild gastritis with mild glandular atrophy, mild infiltration by inflammatory cells, irregular arrangement of surface epithelium, and irregular opening pits (b).

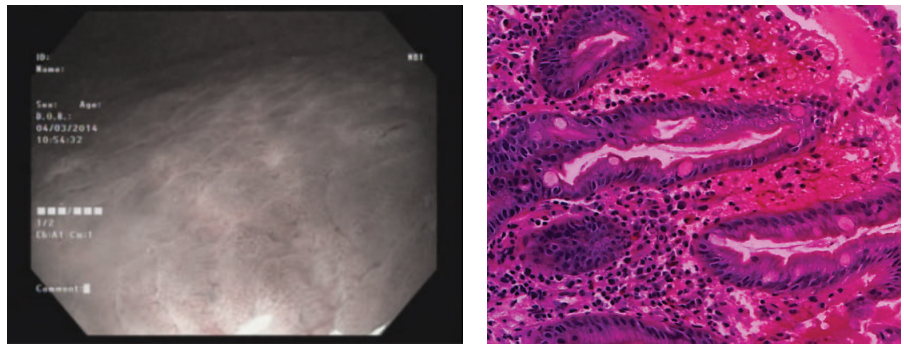


(a) (b)

Figure 5: Type 3 rod-shape gastric pit with prominent sulcus (a). This pattern is associated with moderate glandular atrophy, moderate infiltration by inflammatory cells, and irregular arrangement of surface epithelium (b).

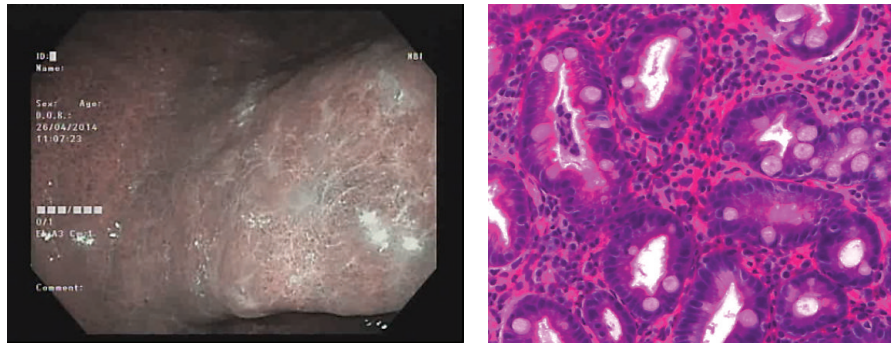
cases were evaluated by 5 pathologists from Bangkok Pathological Laboratory outside Suranaree University according to the Sydney classification (Table 1), including evaluation of chronic inflammation, atrophy, intestinal metaplasia, and activity of gastritis.

2.5. Endoscopic Findings. Local anesthesia was the same as that for conventional gastroscopy. The gastroscopic procedures were performed using an upper GI video endoscope (Olympus EVIS EXERA III, CV-190). The whole stomach was examined first with conventional endoscopy. After the whole



(a) (b)

Figure 6: Type 4 ground glass-like patterns (a). Marked gastritis was found in type 4, with marked glandular atrophy, marked lymphocytic infiltration, lymphoid follicular hyperplasia, and mild intestinal metaplasia (b).



(a) (b)

Figure 7: Type 5 brownish patches with bluish margin and irregular border (a). This pattern corresponded to marked intestinal metaplasia with marked gastric atrophy, indicating advanced gastritis (b).

stomach mucosa was observed we chose site of specific gastric mucosa according to previous studies using magnification endoscopy [11–13]. The observed gastric mucosal morphologic pattern was classified into 5 morphologic patterns: type 1: regular arrangement of collecting venules, type 2: cone-shaped gastric pits, type 3: rod-shaped gastric pits with prominent sulci, type 4: ground glass-like pattern, and type 5: dark brown patches. Types 1 and 2 represent normal and mild inflammation morphologies, and types 3–5 represent moderate to severe inflammation morphologies (Figure 2) [9, 13].

2.6. Image Evaluation. All gastroscopic examinations were digitally recorded and still images of the observation sites were captured for the use in the reproducibility study. The selected images were transferred to a software program without distorting brightness, contrast, or color balance. All endoscopists classified them as type 1 through type 5 gastric mucosal morphologic patterns as described above. A total of 200 pictures from 200 patients were selected for the inter- and intraobserver agreement study. All endoscopists were blinded to the results of the *Helicobacter pylori* status and histology before reviewing the slides.

2.7. Statistical Analysis. The sensitivity, specificity, positive predictive value, and negative predictive values were calculated. Correlation with histopathological severity performs using Chi-square test. A P value of < 0.05 was considered significant. All statistical analyses were performed using the SPSS, version 16.0 (SPSS Inc., Chicago, IL, USA). The κ value was calculated for inter- and intraobserver variability. Interobserver variation was calculated from the results of the first reading, with 3 pairs in all. Intraobserver variation was determined by comparing the first and second assessment of each endoscopist, with 3 pairs in all. κ values below 0.4 indicated poor agreement, values between 0.4 and 0.6 represent moderate agreement, values between 0.6 and 0.8 represented substantial agreement, and values greater than 0.8 corresponded to excellent agreement.

3. Results

A total of 200 consecutive patients (92 men, 118 women; mean age: 49.0 years, range: 19–69 years) were enrolled in the study from January 2014 to November 2014. The 200 patients included 35 patients showing a type 1 pattern, 25 patients showing a type 2 pattern, 29 patients showing a type 3 pattern,

Table 1: Updated Sydney system.

Histologic properties	Definition	Grade		
		Mild	Moderate	Severe
Chronic inflammation	Lymphocyte and plasma cell in lamina propria	1+	2+	3+
Neutrophil activation	Neutrophilic infiltration in lamina propria or superficial epithelium	<1/3	1/3–2/3	>2/3
Glandular atrophy	Loss of corpus and antral glands	1+	2+	3+
Intestinal metaplasia	Intestinal metaplasia of mucosal epithelium	<1/3	1/3–2/3	>2/3
Helicobacter pylori	*Helicobacter pylori* intensity	1+	2+	3+

51 patients showing a type 4 pattern, and 60 patients showing a type 5 pattern (Table 2). *Helicobacter pylori* infection was demonstrated by both a positive result in the rapid urease test and the presence of bacteria seen on histological examination in 134 patients (67%).

Type 1 and type 2 gastric mucosal morphologic patterns were statistically significant in predicting *Helicobacter pylori* negative status as compared with other mucosal morphologic patterns (58/60, $P < 0.01$) (Figure 1). Type 3, type 4, or type 5 gastric mucosal morphologic patterns were statistically significant in predicting *Helicobacter pylori* positive status as compared with other mucosal morphologic patterns (132/140, $P < 0.01$). Furthermore, the sensitivity, specificity, and positive and negative predictive values of type 3, type 4, or type 5 morphologic patterns for predicting the *Helicobacter pylori* positive gastric morphologic patterns were 94.28%, 96.66%, 98.50%, and 87.87%, respectively, with good correlation between gastric mucosal morphologic patterns and the severity of inflammation ($P < 0.01$).

3.1. Gastric Mucosal Morphology and Severity of Gastric Mucosal Inflammation. Type 1 gastric mucosal morphologic patterns were associated with regular arrangement of surface epithelium, with no infiltration by inflammatory cells (Figure 3). Type 2 abnormal C-NBI mucosal morphologic patterns corresponded to mild gastritis with mild glandular atrophy, mild infiltration by inflammatory cells, irregular arrangement of surface epithelium, and irregular opening pits (Figure 4). Moderate gastritis was recognized in type 3, with moderate glandular atrophy, moderate infiltration by inflammatory cells, and irregular arrangement of surface epithelium (Figure 5). Marked gastritis was found in type 4, with marked glandular atrophy, marked lymphocytic infiltration, lymphoid follicular hyperplasia, and mild intestinal metaplasia (Figure 6). The dark brown patches in type 5 corresponded to marked intestinal metaplasia with severe gastric atrophy (Figure 7), indicating advanced gastritis.

3.2. Different C-NBI Gastric Mucosal Morphologies and Correlation with Histopathology

3.2.1. Inter- and Intraobserver Agreement Assessment. The k values for inter- and intraobserver agreement for the gastroscopic mucosal morphologic patterns were significant. The k values for inter- and intraobserver agreement with regard to the prediction of *Helicobacter pylori* infection status were also significant (Table 4).

4. Discussion

Given that evidence from histopathology remains the gold standard for diagnosing *Helicobacter pylori* infection, the reliability of detecting *Helicobacter pylori* associated gastritis and related conditions such as atrophy and intestinal metaplasia by "*random biopsy*" sampling of gastric mucosa largely depends on the site, number, and size of biopsy specimens. Such a practice of random sampling can result in sampling errors, missed pathology, unnecessary work for pathology departments, and increase in costs of investigations. Early diagnosis and eradication of *Helicobacter pylori* infection are a key step in eliminating cancer risk. Real time identification of the area of *Helicobacter pylori* infection in the stomach during gastroscopy not only reduces the sampling error and excessive work load of laboratories but also improves detection efficacy of early gastric malignant lesions albeit this procedure needs more meticulous examination of the whole stomach [11]. The development of high resolution magnifying gastroscopy markedly overcame these problems [12, 14, 15], yet the use of magnified imaging for routine daily screening for *Helicobacter pylori* infection is impractical. It is not only costly but also less widely available, and it takes more time. In addition, it requires special patient preparation and need for experienced endoscopist. In our study, the gastric mucosal morphologic patterns were classified into 5 morphologic patterns using C-NBI gastroscopy. Type 1 and type 2 gastric mucosal morphologic patterns both were statistically significant in predicting *Helicobacter pylori* negative status compared to other gastric mucosal morphologic patterns ($P < 0.01$), whereas type 3, type 4, or type 5 gastric mucosal morphologic patterns were statistically significant in predicting *Helicobacter pylori* positive status as compared to the other gastric mucosal morphologic patterns ($P < 0.01$). These 3 gastric mucosal morphologic patterns (types 3, 4, and 5) were combined for analysis, yielding a good sensitivity, specificity, and positive and negative predictive values (94.28%, 96.66%, 98.50%, and 87.87%, resp.) for diagnosis of *Helicobacter pylori* infection. The Sydney system with the description and classification of histological severity of gastritis has become well established, whereas no international consensus has yet been reached on the description and classification of specific gastroscopic gastritis findings. The present study of the correlation between gastric mucosal morphologic pattern and histological gastritis severity (using the updated Sydney classification) shows a good correlation between the gastric mucosal morphologic pattern and the severity of gastritis ($P < 0.01$).

Table 2: Correlation between gastric mucosal morphologic patterns and *Helicobacter pylori* infection status.

Mucosal morphology	*Helicobacter pylori* infection status		*P*
	Noninfected subjects (HP−)	Infected subjects (HP+)	
Type 1	35 (35/35)*	—	<0.01
Type 2	23 (23/25)*	2 (2/25)	<0.01
Type 3	4 (4/29)	25 (25/29)*	<0.01
Type 4	3 (3/51)	48 (48/51)*	<0.01
Type 5	1 (1/60)	59 (59/60)*	<0.01

*Statistical significant.

Table 3: Correlation between gastric mucosal morphologic patterns and inflammation grading.

Mucosal morphology	Inflammation grading			*P*
	Mild	Moderate	Severe	
Type 1	35 (35/35)	—	—	<0.01
Type 2	10 (10/25)	15 (15/25)	—	<0.01
Type 3	5 (5/29)	24 (24/29)	—	<0.01
Type 4	—	27 (27/51)	24 (24/51)	<0.01
Type 5	—	19 (19/60)	41 (41/60)	<0.01

Table 4: Inter- and intraobserver agreement.

	Interobserver agreement		Intraobserver agreement	
	% agreement	*k* value (95% CI)	% agreement	*k* value (95% CI)
Gastric mucosal morphology	98.8	0.98 (0.97–0.98)	89.9	0.89 (0.87–0.89)
H. pylori infection status	97.9	0.94 (0.92–0.95)	98.3	0.97 (0.96–0.97)

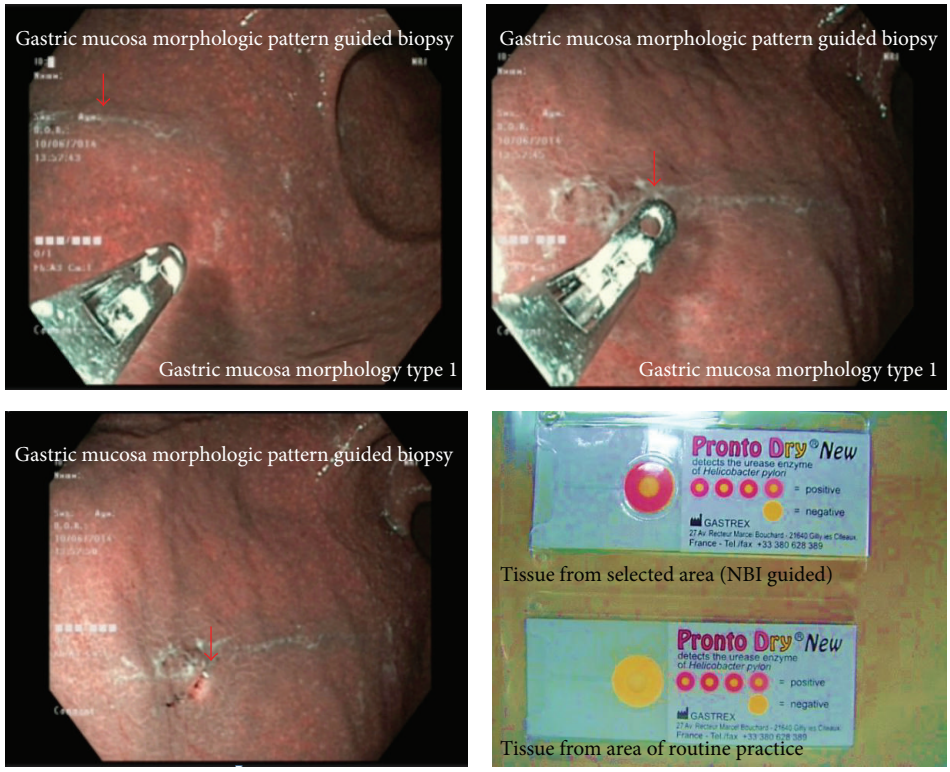


Figure 8: Site specific biopsy.

In conclusion, considering the unsatisfactory sensitivity of conventional gastroscopy for diagnosing *Helicobacter pylori* associated gastritis and the limitations of magnified endoscopic imaging technique, our study suggests that gastric mucosal morphologic pattern in *Helicobacter pylori* infected gastric mucosa can be reliably identified using C-NBI gastroscopy and can also predict the histopathological severity of gastritis.

4.1. Comment and Future Direction. Our study found good correlation between gastric mucosal morphologic pattern, *Helicobacter pylori* status, and severity of pathological inflammation grading (Table 3). Thus gastric mucosal morphologic pattern may be useful for "*site specific biopsy*" of the areas with suspected *Helicobacter pylori* infection in daily practice (Figure 8). We plan to compare the gold standard biopsy technique with site specific biopsy technique in the next study.

Disclosure

This study was part of study about effect of probiotic for eradication of *Helicobacter pylori*.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Comparison of Proton Pump Inhibitor and Histamine-2 Receptor Antagonist in the Prevention of Recurrent Peptic Ulcers/Erosions in Long-Term Low-Dose Aspirin Users: A Retrospective Cohort Study

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Background. Proton pump inhibitor and histamine-2 receptor antagonist can prevent aspirin-related ulcers/erosions but few studies compare the efficacy of these two agents. *Aims*. We evaluated the efficacy of omeprazole and famotidine in preventing recurrent ulcers/erosions in low-dose aspirin users. *Methods*. The 24-week clinical outcomes of the patients using low-dose aspirin for cardiovascular protection with a history of ulcers/erosions and cotherapy of omeprazole or famotidine were retrospectively reviewed. The incidence of gastrointestinal symptoms, recurrent ulcers/erosions, erosive esophagitis, gastrointestinal bleeding, and thromboembolic events was analyzed. *Results*. A total of 104 patients (famotidine group, 49 patients; omeprazole group, 55 patients) were evaluated. Famotidine group had more gastrointestinal symptoms episodes than omeprazole group (46.9% versus 23.6%, $P = 0.01$). Fifteen famotidine group patients and 5 omeprazole group patients had recurrent ulcers/erosions (30.6% versus 9.1%, $P = 0.005$). Lanza scale was significantly lower in omeprazole group than in famotidine group (1.2 ± 0.7 versus 1.7 ± 1.1, $P = 0.008$). Only 1 famotidine group patient had ulcer bleeding. The incidences of erosive esophagitis and thromboembolic events were comparable between both groups. *Conclusions*. Omeprazole was superior to famotidine with less gastrointestinal symptoms and recurrent ulcers/erosions in patients using 24-week low-dose aspirin. The risk of erosive esophagitis, gastrointestinal bleeding, and thromboembolic events was similar between both groups.

1. Introduction

Aspirin is a nonsteroidal anti-inflammatory drug (NSAID) with antiplatelet effect and has been widely used in primary or secondary prevention of cardiovascular and cerebrovascular events at a low dose of 75 to 325 mg daily [1–3]. Low-dose aspirin reduces prostaglandin levels in stomach and duodenum [4] and induces gastric and duodenal mucosal injury, ulcer formation, or bleeding [5, 6]. Proton pump inhibitors (PPI) have gastroduodenal protective effect of aspirin-related ulcers [7]. To prevent mucosa damage caused by aspirin, ACC/AHA guideline suggests cotherapy of aspirin and PPI after unstable angina or non-ST elevation myocardial infarction in patients with previous gastrointestinal bleeding (GIB) [8]. Recently, a nationwide cohort study in Taiwan also found that aspirin plus PPI was superior to clopidogrel alone or clopidogrel plus PPI in terms of reducing the risk of GIB while maintaining the cardiovascular protective effect [9].

However, long-term PPI use may be associated with some adverse effects. Increased risk of osteoporosis and fracture is observed in patients using long-term PPIs [10]. Besides, patients using long-term PPIs are at a risk of hypergastrinemia, hypochlorhydria, hypomagnesaemia, malabsorption, and infectious diarrhea [11]. The safety of long-term PPIs should be concerned in aspirin users in addition to the gastroduodenal protective effect of PPIs.

Helicobacter pylori (*H. pylori*) infection increases the risk of peptic ulcer diseases in patients using NSAIDs including aspirin [12]. *H. pylori* eradication can prevent gastropathy and should be undertaken in patients with a history of peptic ulcers and who need long-term aspirin therapy [13]. After eradication of *H. pylori*, cotherapy with a PPI is still suggested in high-risk aspirin users [13]. However, whether use of other acid suppressants such as a histamine-2 receptor antagonist (H2RA) is feasible in this case is seldom investigated.

H2RAs had also been proved to reduce the gastric toxicity of aspirin [14]. Although PPIs have a stronger acid suppression capacity than H2RAs, patients using H2RAs are at a lower risk of pneumonia and *Clostridium difficile* infection than PPIs users [15]. The efficacy of PPIs and H2RAs in the prevention of recurrent ulcers/erosions in aspirin users is rarely compared. Omeprazole and famotidine are both potent acid suppressants which decrease the incidence of recurrent ulcers/erosions in patients taking long-term aspirin [14, 16, 17]. This study aimed at comparing the protective effect of omeprazole and famotidine in long-term low-dose aspirin users with a history of aspirin-related peptic ulcers. All the patients with *H. pylori* infection in this cohort received eradication therapy of *H. pylori* as suggested by the guidelines.

2. Materials and Methods

2.1. Patients. This retrospective cohort study was conducted at Kaohsiung Veterans General Hospital and was approved by the Institutional Review Board (VGHKS13-CT12-07). We reviewed the medical records of the patients using long-term low-dose aspirin from January 2008 to December 2012. Patients were considered to be enrolled into this study if they met the following inclusion criteria: (1) age equal to or more than 20 years, (2) use of aspirin 75 to 325 mg daily for primary or secondary prevention of coronary artery disease or cerebral vascular accident, (3) a history of aspirin-related peptic ulcers/erosions, (4) no gastroduodenal ulcers/erosions or erosive esophagitis on esophagogastroduodenoscopy (EGD) at the initial analysis point, (5) concomitant use of famotidine 40 mg daily or omeprazole 20 mg daily for the prevention of recurrent aspirin-related ulcers, (6) undergoing EGD at around 24 weeks from the initial analysis point, and (7) being negative for *H. pylori* infection by a histology, rapid urease test, or urea breathing test at the initial analysis point. The exclusion criteria were (1) active malignancy; (2) a history of surgery for esophagus, stomach, or duodenum; (3) concomitant use of anticoagulants, thienopyridines, misoprostol, antacid, and mucosa protecting agents; (4) use of NSAIDs ≥1 week; (5) use of aspirin, famotidine, or omeprazole for less than 6 months from the initial analysis point; (6) pregnancy; and (7) chronic renal insufficiency.

2.2. Methods. According to the standard treatment in our institute, patients with a peptic ulcer/erosion history who underwent long-term aspirin therapy would receive concomitant PPI or H2RA therapy. Additionally, follow-up endoscopy was performed 6 months later and whenever severe dyspepsia or GIB occurred and *H. pylori* testing was also conducted. In this study, we retrospectively reviewed the demographic data of the patients including age, gender, personal habits (cigarette, alcohol, coffee, and tea consumption), concomitant diseases, indications of aspirin use, doses of aspirin, concomitant medications including steroids and short-term (<1 week) NSAIDs during the study period, history of upper GIB if present, history of *H. pylori* infection, and the eradication therapies of *H. pylori*, and the findings of follow-up EGD were retrieved from the medical records. Besides, the incidences of gastrointestinal symptoms including epigastralgia, bloating, nausea and vomiting, recurrent peptic ulcers/erosions, erosive esophagitis, peptic ulcer bleeding, cerebral vascular accident, transient ischemic attack, and acute coronary syndrome were assessed.

2.3. Definition. An ulcer was defined as a mucosal break more than or equal to 3 mm of its longest diameter found on EGD and was estimated by biopsy forceps. An erosion was defined as a mucosal break less than 3 mm of its longest diameter. The severity of gastroduodenal mucosal injury was estimated using Lanza scale (grade 0: no visible lesions; grade 1: mucosal hemorrhage only (≤25); grade 2: 1-2 erosions, or >25 hemorrhages; grade 3: 3–9 erosions; grade 4: ≥10 erosions or an ulcer) [18]. Consumption of coffee, tea, and alcohol was defined as consumption of coffee, tea, or alcohol more than or equal to 4 days per week. Cigarette consumption was defined as daily smoking during the study period. *H. pylori* infection status was determined by rapid urease test, urea breath test, or histology results at the initial analysis point. Upper GIB was defined as patients presented with hematemesis, tarry stool, or hematochezia or bleeding found on endoscopic examination. Acute coronary syndrome was defined as occurrence of unstable angina or acute myocardial infarction. Cerebral vascular accident was defined as patients presenting with typical neurological symptoms such as hemiplegia, dysphagia, and slurred speech or typical image findings. Transient ischemic attack was defined as patients presented with typical neurological symptoms and fully recovered within 24 hours.

2.4. Study End Points. The primary end point of this study was recurrent ulcers or erosions found on endoscopic examination. The secondary end points were occurrence of gastrointestinal symptoms, erosive esophagitis, upper GIB, and thromboembolic events including acute coronary syndrome, ischemic stroke, or transient ischemic attack.

2.5. Statistical Analysis. Demographic data and the occurrence of primary and secondary end points were compared between both groups. Categorical data were compared using

Table 1: Demographic data of patients taking long-term low-dose aspirin.

Variables	Famotidine group ($n = 49$)	Omeprazole group ($n = 55$)	P value
Age (year)	74.4 ± 10.5	73.3 ± 10.6	0.6
≥60 years old	44 (89.8%)	48 (87.3%)	0.7
Male gender	42 (85.7%)	42 (76.4%)	0.2
Time to follow-up EGD* (weeks)	25.0 ± 2.3	24.5 ± 2.1	0.2
Smoking	6 (12.2%)	8 (14.5%)	0.7
Alcohol consumption	2 (4.1%)	3 (5.5%)	1.0
Coffee consumption	4 (8.2%)	7 (12.7%)	0.4
Tea consumption	15 (28.6%)	8 (14.5%)	0.8
Cirrhosis	1 (2.0%)	1 (1.8%)	1.0
Hiatus hernia	18 (36.7%)	18 (32.7%)	0.7
History of upper GIB†	10 (20.4%)	12 (21.8%)	0.9
History of erosive esophagitis	8 (16.3%)	17 (30.1%)	0.08
History of *H. pylori* infection	19 (38.8%)	14 (25.5%)	0.1
Concomitant medication			
Steroids	3 (6.1%)	2 (3.6%)	0.6
Short-term NSAID‡	5 (10.2%)	7 (12.7%)	0.7

*EGD: esophagogastroduodenoscopy.
†GIB: upper gastrointestinal bleeding.
‡NSAID: nonsteroidal anti-inflammatory drug.

Chi-square or Fisher's exact tests when appropriate. Continuous variables with normal distributions were compared using independent Student's t-test. Continuous variables without normal distributions were compared using Mann-Whitney U test. Univariate logistic regression analysis was performed to examine the variables significantly associated with recurrent peptic ulcers or erosions. Significance was defined as $P < 0.05$ for all two-tailed tests. All analyses were conducted by using SPSS software (version 12; SPSS Inc., Chicago, IL).

3. Results

3.1. Characteristics of Patients. Between January 2008 and December 2012, a total of 104 eligible patients (famotidine group, 49 patients; omeprazole group, 55 patients) using long-term low-dose aspirin were analyzed. All the patients received routine *H. pylori* testing because they had a history of aspirin-related peptic ulcers/erosions. Of the 19 famotidine group patients with a history of *H. pylori* infection, *H. pylori* was successfully eradicated by using 7-day triple therapy (PPIs, amoxicillin, and clarithromycin for 7 days) (11 patients) and 14-day hybrid therapy (PPIs and amoxicillin for 14 days plus clarithromycin and metronidazole for 7 days) (8 patients). Of the 14 omeprazole group patients with a history of *H. pylori* infection, *H. pylori* was successfully eradicated by using 7-day triple therapy (5 patients) and 14-day hybrid therapy (9 patients). All the patients were negative for *H. pylori* infection by a histology, rapid urease test, or urea breathing test at the initial analysis point. All the patients with a history of *H. pylori* infection were still negative for *H. pylori* testing in the endpoint of analysis. The demographic data of famotidine group and omeprazole group showed similar age, gender, duration from the initial analysis point to follow-up endoscopy, and consumption of smoking, alcohol, coffee, and tea. The proportions of history of upper GIB, history of *H. pylori* infection, indications of aspirin therapy, and concomitant use of short-term NSAIDs and steroids were also comparable between both groups (Table 1).

3.2. Incidence of Gastrointestinal Symptoms and Bleeding. Twenty-three episodes of gastrointestinal symptoms were observed in famotidine group: dyspepsia (8 patients), acid reflux (8 patients), epigastralgia (6 patients), and belching (1 patient). One patient in famotidine group presented with gastric ulcer bleeding (Table 2). Thirteen episodes gastrointestinal symptoms were found in omeprazole group patients: dyspepsia (5 patients), acid reflux (2 patients), epigastralgia (5 patients), and belching (1 patient). No patient in omeprazole group had GIB. Significantly more episodes of gastrointestinal symptoms were found in famotidine group patients than in omeprazole group patients (46.9% versus 23.6%, $P = 0.01$).

3.3. Findings of Follow-Up Endoscopy. Patients in famotidine group had a significantly higher incidence of recurrent ulcers/erosions than patients in omeprazole group (30.6% versus 9.1%, $P = 0.005$). Of the patients with recurrent ulcers/erosions, asymptomatic ulcers/erosions were found in 9 of 15 (60.0%) famotidine group patients and 4 of 5 (80.0%) omeprazole patients. Five of 19 patients (26.3%) with a history of *H. pylori* infection in famotidine group had recurrent ulcers/erosions and 1 of 14 patients (7.1%) with a history of *H. pylori* infection in omeprazole group had recurrent ulcers/erosions ($P = 0.2$). In ulcer analysis, 10 patients in famotidine group (20.4%) and 4 patients in omeprazole group (5.5%) had recurrent ulcers ($P = 0.04$). We evaluated the severity of gastroduodenal mucosal injury using Lanza score and found that omeprazole also showed a better protective effect than famotidine (1.2 ± 0.7 versus

Table 2: Sequelae of patients taking long-term low-dose aspirin.

	Famotidine group ($n = 49$)	Omeprazole group ($n = 55$)	P value
Gastrointestinal symptoms	23 (46.9%)	13 (23.6%)	0.01
Dyspepsia	8 (16.3%)	5 (9.1%)	0.3
Acid reflux	8 (16.3%)	2 (3.6%)	0.04
Epigastralgia	6 (12.2%)	5 (9.1%)	0.6
Belching	1 (2%)	1 (1.8%)	1.0
Peptic ulcer bleeding	1 (2%)	0 (0%)	0.5

Table 3: Follow-up endoscopic findings of patients taking long-term low-dose aspirin.

	Famotidine group ($n = 49$)	Omeprazole group ($n = 55$)	P value
Lanza scale	1.7 ± 1.1	1.2 ± 0.7	0.008
Gastroduodenal ulcer/erosion*	15 (30.6%)	5 (9.1%)	0.005
Ulcer	10 (20.4%)	3 (5.5%)	0.04
Gastric ulcer†	6	2	
Duodenal ulcer	3	1	
Gastric ulcer & duodenal ulcer	1	0	
Erosion	5 (10.2%)	2 (3.6%)	0.2
Gastric erosion	5	1	
Gastric erosion & duodenal erosion	0	1	
Erosive esophagitis	7 (14.3%)	7 (12.7%)	1.0

*Results were presented as the most severe mucosal injury found on endoscopy.
†One patient in famotidine group had gastric ulcer bleeding.

1.7 ± 1.1, $P = 0.008$). Besides, erosive esophagitis was present in 7 patients (14.3%) in famotidine group and 7 patients (12.7%) in omeprazole group ($P = 1.0$) (Table 3).

Univariate logistic regression analysis of variables including age ≥60 years, gender, indications of aspirin use, history of upper GIB, history of *H. pylori* infection, use of NSAIDs <1 week, and steroids therapy found that omeprazole therapy was the only factor associated with a lower risk of aspirin-related ulcers/erosions (relative risk: 0.2; 95% confidence interval: 0.08–0.7; $P = 0.008$) (Table 4).

3.4. Incidence of Thromboembolism. In omeprazole group, 4 patients had acute coronary syndrome (unstable angina, 2 patients; acute myocardial infarction, 2 patients) while no patient in famotidine group had thromboembolic event. The incidence of thromboembolic events was comparable between both groups (Table 5).

4. Discussion

The current study revealed that omeprazole was superior to famotidine in the prevention of recurrent ulcers/erosions in long-term low-dose aspirin users free for active *H. pylori* infection. A very low upper GIB rate was observed in this cohort. Famotidine group patients had more episodes of gastrointestinal symptoms than omeprazole group patients. Besides, the incidences of erosive esophagitis and thromboembolic events were similar between omeprazole and famotidine group patients.

Few studies compared the efficacy of PPIs and H2RAs in the prevention of recurrent peptic ulcers due to long-term aspirin use. Ng et al. found that high-dose famotidine (80 mg daily) was inferior to pantoprazole (20 mg daily) with a significantly higher incidence of aspirin-related ulcers/erosions or GIB [19]. However, only patients with dyspepsia, severe epigastric pain, or GIB underwent endoscopy and the incidence of asymptomatic ulcers/erosions might be underestimated. Tamura et al. evaluated patients taking low-dose aspirin with either standard-dose famotidine (40 mg daily) or lansoprazole (15 mg daily). Significantly, more patients in famotidine group (48.4%) presented with gastroduodenal erosions than patients in lansoprazole group (17.0%) and no ulcer was found in both groups [20]. Nevertheless, the treatment duration of acid suppressants was not identical between both groups. Besides, part of the patients took antiplatelet agents other than aspirin or anticoagulants and nearly half of the patients were positive for urinary *H. pylori* antibody while only about 10% of the patients had received eradication therapy of *H. pylori*. The incidence of aspirin-related ulcers might be overestimated because of high *H. pylori* infection rate and concomitant use of antiplatelet agents or anticoagulants in some patients. Another study by Ng et al. found that esomeprazole was superior to famotidine in preventing upper GIB, perforation, or obstruction from ulcers/erosions in patients with acute coronary syndrome or myocardial infarction [21]. Unfortunately, a combination of aspirin, clopidogrel, and enoxaparin or thrombolytics was used and the role of aspirin was not specifically investigated.

This study compared the protective efficacy of standard-dose famotidine with omeprazole after long-term low-dose aspirin use with the superiority that all patients were free for active *H. pylori* infection and all patients underwent

Table 4: Univariate analysis of risk factors for recurrent ulcers/erosions in long-term low-dose aspirin users.

Variables	Relative risk	95% confidence interval	*P* value
Age ≥ 60 years	0.4	0.1–1.6	0.2
Primary prevention	1.1	0.4–3.3	0.8
Male gender	2.5	0.5–11.6	0.3
Omeprazole group	0.2	0.08–0.7	0.008
Smoking	2.8	0.8–9.5	0.1
Alcohol consumption	3.0	0.5–19.3	0.2
Coffee consumption	0.4	0.05–3.2	0.4
Tea consumption	1.3	0.4–4.1	0.6
History of UGIB*	0.6	0.2–2.3	0.5
History of *H. pylori* infection	0.9	0.3–2.6	0.9
Concomitant medication			
Steroids	1.1	0.1–10.0	1.0
Use of NSAID† <1 week	1.5	0.4–6.0	0.6

*UGIB: upper gastrointestinal bleeding.
†NSAID: nonsteroidal anti-inflammatory drug.

Table 5: Thromboembolic events in patients taking long-term low-dose aspirin.

	Famotidine group ($n = 49$)	Omeprazole group ($n = 55$)	*P* value
Total events	0	4 (7.3%)	0.1
Acute coronary syndrome	0	4	
Acute myocardial infarction	0	2	
Unstable angina	0	2	
Cerebral vascular accident	0	0	
Transient ischemic attack	0	0	

follow-up endoscopy at the end of the study, which allowed us to estimate the exact incidence of aspirin-related erosions/ulcers. Patients in omeprazole group (9.1%) had a significantly lower incidence of ulcers/erosions compared with patients in famotidine group (30.6%), which was consistent with previous studies. The severity of gastroduodenal injury determined by using Lanza score was also less severe in omeprazole group than in famotidine group. During subgroup analysis, we found that the incidence of gastroduodenal ulcers in omeprazole group (5.5%) was significantly lower than that of famotidine group (20.4%). Our findings supported the ACC/AHA guideline that cotherapy of aspirin and PPI is the treatment of choice in patients with a history of peptic ulcer and who need long-term aspirin therapy.

Gastrointestinal mucosal injury associated with low-dose aspirin is often asymptomatic [22]. Although famotidine group patients had more frequent gastrointestinal symptoms than omeprazole group patients, 65% of the ulcers/erosions found on endoscopy were asymptomatic in this study. Our findings suggested that typical peptic ulcer symptoms such as dyspepsia and epigastralgia were not reliable predictors for recurrent gastroduodenal ulcers/erosions in low-dose aspirin users who were on concomitant acid suppressants. Actually, asymptomatic gastroduodenal erosions also can be associated with GIB [23]. Therefore, periodical endoscopy surveillance might be necessary in patients taking long-term low-dose aspirin even if concomitant acid suppressants are used.

The risk of GIB was very low and similar between patients using omeprazole (0%) and famotidine (2%) in our cohort. This result contradicted the study by Ng et al. in which 5 patients (7.7%) using famotidine had bleeding ulcer/erosion at 9, 2, 32, 16, and 36 weeks, respectively, while no patients using pantoprazole had ulcer/erosion bleeding [19]. It was possible that the study period of our study was 24 weeks and the incidence of GIB might increase after a longer observation period, especially in the patients using famotidine.

The risk of erosive esophagitis is not as high as that of peptic ulcers in aspirin users [24]. Nevertheless, erosive esophagitis is common in low-dose aspirin users with upper GIB [25]. In long-term aspirin user, erosive esophagitis was found in 4.4% of the patients taking famotidine compared with 19.0% of the patients taking placebo [14]. Rabeprazole can also reduce the risk of erosive esophagitis in aspirin user [26]. In this study, the incidence of erosive esophagitis was about 10% in both groups. However, we found that omeprazole group was associated with less incidence of acid reflux, the typical symptom of erosive esophagitis, than famotidine group although the case numbers were limited. A randomized control trial with a larger sample size is necessary to investigate the difference of incidence of erosive esophagitis between PPIs and H2RAs in long-term aspirin users.

For aspirin users, eradication of *H. pylori* can decrease the risk of recurrent ulcer bleeding to a very low extent [27].

Unfortunately, *H. pylori* eradication alone does not reduce the incidence of ulcers in patients already receiving long-term aspirin and continued PPI treatment is still necessary [13]. In our study, the incidence of recurrent erosions/ulcers was similar between omeprazole and famotidine group during subgroup analysis of the patients receiving eradication therapy of *H. pylori* but the power was limited. Further study is required to compare the efficacy of PPIs and H2RAs in the prevention of recurrent aspirin-related ulcers after eradication of *H. pylori*.

There were some limitations in our study. First, selection bias and missing data did exist in this retrospective cohort study and the final results might be biased. Second, although omeprazole patients had less gastrointestinal symptoms than famotidine group patients, the symptoms might be missed if they were not documented by the caring physicians. Third, the study period was only 24 weeks and a higher incidence of gastroduodenal ulcers/erosions would be expected after a longer observation period. Fourth, we could not exclude the possibility of false-negative *H. pylori* testing in omeprazole group patients even though all patients were negative for active *H. pylori* infection during the study period. Finally, although the risk of GIB and thromboembolic event was similar between omeprazole and famotidine group, the sample size of this cohort was not large enough and further studies are required to compare the incidence of GIB and thromboembolism between PPI and H2RA users.

5. Conclusions

In conclusion, this study suggested that cotherapy of omeprazole had less gastrointestinal symptoms and a better protective efficacy in the prevention of recurrent peptic ulcers/erosions than famotidine in patients using long-term low-dose aspirin. However, the risk of erosive esophagitis, GIB, and thromboembolic events was similar between these two therapeutic strategies in this cohort. Because of the high occurrence rate of asymptomatic ulcers/erosions, periodical endoscopic surveillance might be necessary in patients using cotherapy of acid suppressants and long-term low-dose aspirin.

Abbreviations

PPI:	Proton pump inhibitor
H2RA:	Histamine 2 receptor antagonist
GIB:	Gastrointestinal bleeding
NSAID:	Nonsteroidal anti-inflammatory drug
H. pylori:	*Helicobacter pylori*
EGD:	Esophagogastroduodenoscopy.

Conflict of Interests

The authors declare that the study was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interests.

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Review Article

Quinolone-Containing Therapies in the Eradication of *Helicobacter pylori*

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Fluoroquinolones, especially levofloxacin, are used in the eradication of *Helicobacter pylori* worldwide. Many consensus guidelines recommend that the second-line rescue therapy for *H. pylori* eradication consists of a proton pump inhibitor, a quinolone, and amoxicillin as an option. Unfortunately, quinolone is well associated with a risk of developing bacterial resistance. In this paper, we review quinolone-containing *H. pylori* eradication regimens and the challenges that influence the efficacy of eradication. It is generally suggested that the use of levofloxacin should be confined to "rescue" therapy only, in order to avoid a further rapid increase in the resistance of *H. pylori* to quinolone. The impact of quinolone-containing *H. pylori* eradication regimens on public health issues such as tuberculosis treatment must always be taken into account. Exposure to quinolone is relevant to delays in diagnosing tuberculosis and the development of drug resistance. Extending the duration of treatment to 14 days improves eradication rates by >90%. Tailored therapy to detect fluoroquinolone-resistant strains can be done by culture-based and molecular methods to provide better eradication rates. Molecular methods are achieved by using a real-time polymerase chain reaction to detect the presence of a *gyrA* mutation, which is predictive of treatment failure with quinolones-containing triple therapy.

1. Introduction

The rate of eradication obtained using a triple therapy approach has decreased substantially for the first- and second-line regimens in recent years, owing to an increasing rate of antibiotic resistance [1]. Fluoroquinolones, especially levofloxacin, have been widely used to eradicate *Helicobacter pylori* worldwide [2]. The American College of Gastroenterology Guideline on the Management of *Helicobacter pylori* Infection [3], the second Asia Pacific consensus guidelines for *Helicobacter pylori* infection [4], and the Maastricht IV/Florence-Consensus Report [5] recommend that second-line *H. pylori* eradication rescue therapy consists of a PPI, a quinolone, and amoxicillin as an option. However, antibiotic resistance is one of the key factors responsible for failure of eradication of *H. pylori*, as well as poor compliance, high gastric acidity, a high bacterial load, and cytochrome P450 2C19 (CYP2C19) polymorphism [2, 6]. Unfortunately, quinolone is well associated with a risk of developing resistant bacterial strains [7]. Here we review fluoroquinolone-based *H. pylori* eradication regimens and discuss the challenges we are faced with owing to the emerging resistance to antibiotics that can influence the efficacy of eradication, particular the public health issue of tuberculosis.

Levofloxacin is a levorotatory isomer of ofloxacin with known activity against many Gram-negative and Gram-positive bacteria. The mode of action of levofloxacin is based on the inhibition of bacterial DNA topoisomerase II [8]. The advantage of levofloxacin-containing triple therapy is that there is an *in vivo* synergistic effect with respect to quinolone antimicrobial agents and proton-pump inhibitors (PPIs) when strains of *H. pylori* are targeted [9]. The prevalence of

resistant strains is variable in different geographic areas. For example, there was zero resistance to levofloxacin in Malaysia but 8.2% resistance in Japan [10, 11]. On the other hand, increasing primary levofloxacin resistance has been reported worldwide because of plasmid-mediated horizontally transferable genes encoding quinolone resistance (18.4% in Vietnam, 20.6% in China, 63.3% in Pakistan, 29.1% in Germany, 33.9% in Portugal, 19% in Alaska, and 23% in Brazil) [12–19]. An increased use of quinolones in various different countries is probably responsible for this rise in quinolone resistance across different classes of bacteria, including *H. pylori*. Therefore, it is suggested that the use of levofloxacin should be confined to "rescue" therapy only, in order to avoid a further rapid increase in the resistance of *H. pylori* to quinolone [2]. One of our previous publications reported that quinolone therapy is effective when used to treat a susceptible infection but should be avoided when resistance is present [20].

2. Quinolone-Containing First-Line *H. pylori* Eradication

It is recommended that the standard triple therapy should now be avoided in areas where clarithromycin resistance is high (>15–20%) [5]. A prolonged duration to 14 days of the standard clarithromycin-based triple therapy improved the eradication rate to 82.2% but was still not good enough to attain a grade A or B report card [21]. Because of its ability to overcome metronidazole resistance, the 10-day bismuth-containing quadruple therapy could be an alternative in areas with a high prevalence of clarithromycin and metronidazole resistance but is associated with poor compliance due to side effects [22].

In sequential therapy, patients are prescribed with 5 days' dual therapy with a PPI (standard dose, b.i.d.) and amoxicillin (1000 mg, b.i.d.), followed by 5 days' triple therapy with a PPI (standard dose, b.i.d.), clarithromycin (500 mg, b.i.d.), and metronidazole (500 mg, b.i.d.) [23, 24]. At the beginning, it has been proven to be able to attain a >90% in many studies in Europe and Asia, for instance, 97% in Italian populations and 95.2% in Hong Kong [25–27], but the recent data from other countries appeared to be less effective in countries such as Korea (86.4%) and Iran (88.7%) [28, 29]. The results were even unacceptable in Latin American (76.5%) and Thailand (57.1) [21, 30].

Other alternatives include concomitant therapy and hybrid therapy, which provide >90% eradication rates even in areas with high rates of clarithromycin and metronidazole resistance. Concomitant therapy consists of a PPI (standard dose, b.i.d.) combined with clarithromycin (500 mg, b.i.d.), amoxicillin (1 g, b.i.d.), and metronidazole (500 mg, b.i.d.), prescribed all together at the same time for 7–10 days [31, 32]. It is more convenient than sequential therapy because of the shorter duration of treatment and less complex drug administration. Hybrid therapy has two phases: dual therapy with a PPI (standard dose, b.i.d.) and amoxicillin (1 g, b.i.d.) for 7 days, followed by a non-bismuth quadruple therapy consisting of a PPI (standard dose, b.i.d.), amoxicillin (1 g, b.i.d.), clarithromycin (500 mg, b.i.d.), and metronidazole (500 mg, b.i.d.) for a further 7 days. The benefit of the extended duration of amoxicillin administration is to further reduce the bacterial load to improve the eradication rate [33].

Many clinical trials chose levofloxacin in place of clarithromycin as an alternative first-line regimen. The reported eradication rates varied from 72% to 96% [34, 35]. In a recently published meta-analysis, seven trials were identified with 888 patients receiving 7 days of first-line levofloxacin and 894 treated with standard therapy (Amoxicillin, Clarithromycin and proton pump inhibitor) for 7 days. The overall crude eradication rate in the Levofloxacin group was 79.05% versus 81.4% in the standard group (risk ratio 0.97; 95% CI; 0.93, 1.02) [36]. In another meta-analysis, it was found that eradication rate in the levofloxacin-based therapy group was slightly higher than that in the standard triple therapy group regardless of treatment duration (80.2% versus 77.4%, RR = 1.03, 95% CI = 0.94–1.13) [37]. Subgroup analysis related to different geographic areas found that efficacy of 7-day standard triple regimen was statistically superior to 7-day levofloxacin-based scheme in Asian group (RR = 0.91, 95% CI = 0.86–0.97), but levofloxacin-based triple therapy was predominant regardless of treatment time in European countries (RR = 1.15, 95% CI = 1.06–1.23). It suggests that the 10-day levofloxacin-based triple therapy may be considered as an alternative for increasing cure rate of *H. pylori* infection in European areas. But in many Asian countries, standard triple regimen is still superior to levofloxacin-based therapy as first-line regimen for *H. pylori* eradication. Overall, it appeared that levofloxacin-containing triple therapy as first line regimen was not superior to standard triple therapy and both did not attain a >90% report card.

In sequential therapy the replacement of clarithromycin by levofloxacin offered an equal or better eradication rate [38], but as mentioned previously, the rapid rise in levofloxacin-resistant strains accounted for the failure of eradication. Therefore, levofloxacin-based therapy was no longer recommended as a first-line regimen.

3. Rescue Second-Line Quinolone-Containing Therapy

When first-line therapy fails, the Maastricht IV Consensus Report recommends that the bismuth-containing quadruple therapy is a choice for second-line therapy [5]. However, in areas where bismuth is not available, a levofloxacin-containing triple therapy is recommended. Gisbert and De La Morena reported that a levofloxacin-containing therapy was borderline significant (81%) compared to bismuth-based quadruple therapy (70%) [39]. Extending the duration of treatment has been confirmed to improve the eradication rate. They also confirmed that the 7-day regimen was sub-optimal in terms of treatment duration, and that a longer duration, for example, 10 days, might improve the eradication rate [39]. However, all the studies that used quinolone-containing triple therapy have shown that neither the 7-day nor the 10-day course is able to obtain an eradication rate >90%. Studies of 14 days' quinolone treatment were able to show an eradication rate >90% [7, 20, 40], but again, a

possible increase in quinolone resistance with lengthy use is a major concern.

4. Rescue Third-Line Quinolone-Containing Therapy

The Maastricht IV Consensus Report recommended a selection of antibiotics for third-line regimens, depending on bacterial culture results and antimicrobial sensitivity tests [5]. A report revealed that antimicrobial sensitivity testing in patients who encountered two eradication failures showed the percentage resistance to metronidazole, clarithromycin, levofloxacin, and tetracycline to be 100%, 95%, 31%, and 5%, respectively, and they managed an eradication rate of 90% in the patients they treated by culture-guided therapy [41].

Tailored therapy according to antibiotic resistance has been proposed for achieving a high eradication rate. *H. pylori* antibiotic resistance can be classified into primary, which means there is no previous treatment for eradication of the bacterium and secondary, where a susceptible strain acquires resistance during treatment [42]. The main reasons for this phenomenon are point mutations of *H. pylori* DNA or inappropriate frequent antibiotic use [43, 44]. Tailored therapy to detect quinolone-resistant strains could offer better eradication with quinolone-containing *H. pylori* regimens. Resistances are currently detected by culture-based and molecular methods, but culture-based antibiotic sensitivity testing by *E*-test is time-consuming, and the culture rate of *H. pylori* is approximately 70–80% [45]. The same disadvantage applies to other culture-based tests, such as the agar dilution method, the breakpoint susceptibility test, and the modified disk diffusion method. Moreover, *in vitro* antimicrobial sensitivity testing does not guarantee successful eradication *in vivo*. Therefore, several attempts have been made to substitute for ineffective cultures. One of these is the use of molecular methods, such as real-time polymerase chain reaction (PCR), which can detect the existence of point mutations on quinolone resistance in *H. pylori* (N87 and D91) in the quinolone resistance-determining region of the *gyrA* gene of *H. pylori* [42]. This can be done by using gastric biopsy specimens, which can rapidly provide a >93% success rate [46–48]. The presence of a *gyrA* mutation is predictive of treatment failure with triple therapy for quinolones such as levofloxacin [45]. The advantages of this method are that there is no need for culture; the results are obtained within a few hours; it is commercially available; and it is possible to detect mutations from feces, which means that endoscopy can be avoided [48]. The disadvantages are that each mutation connected to variable antibiotic resistance needs to be determined, and that the cost may be high.

5. The Impact of Quinolone Exposure on Tuberculosis

Unlike other antibiotics used as empirical treatment for community-acquired pneumonia, the quinolones have excellent activity against *Mycobacterium tuberculosis*. In a recent study, researchers found that patients recently exposed to 5 days or more of quinolone were less likely to be smear positive (OR 0.27, 95% CI 0.11 to 0.63), with an increased time to accurate tuberculosis treatment (time ratio 2.02, 95% CI 1.19 to 3.44) [49]. Furthermore, quinolone exposure for >10 days that occurred >60 days before a diagnosis of tuberculosis was associated with the highest risk of quinolone resistance (OR 17.0, 95% CI 5.1–56.8) compared to no exposure [50]. These studies highlighted the important issue that quinolone exposure is relevant to delays in diagnosing tuberculosis and the development of drug resistance. This is particularly important for doctors to bear in mind, especially among certain subsets of patients, such as those infected with human immunodeficiency virus (HIV), or in a country burdened with a high prevalence of tuberculosis such as Taiwan [51]. This could be a clinical challenge when treating *H. pylori* using an extended-duration quinolone-containing triple therapy.

There is a paucity of clinical evidence supporting the hypothesis that the use of quinolone leads to delays in treating tuberculosis in patients with *H. pylori* infection. The clinical impact of the extensive prescription of quinolones for patients with *H. pylori* infection worldwide highlighted the relationship between prior quinolone use and the subsequent emergence of quinolone resistance in *M. tuberculosis* or the delayed diagnosis of tuberculosis.

Gemifloxacin, a newer quinolone with poor activity against *M. tuberculosis* compared to levofloxacin and moxifloxacin, may be a promising alternative to overcome this problem. A dramatic increase in levofloxacin resistance after treatment failure with levofloxacin-containing triple therapy has been found in various different countries. One may need to choose a more potent quinolone in order to prevent the development of quinolone resistance during anti-*H. pylori* therapy. One recent study from Taiwan showed that gemifloxacin was superior to levofloxacin in antimicrobial activity against *H. pylori* isolates and even overcame some levofloxacin resistance [52]. Gemifloxacin is a powerful potent quinolone against *H. pylori*. It should be noted that gemifloxacin exposure is not associated with delay in tuberculosis treatment, and this has been validated in a clinical setting [53]. As a result, gemifloxacin may be the preferred quinolone for treating *H. pylori*, to alleviate any concerns about delaying tuberculosis treatment.

6. Conclusions

The use of quinolones such as levofloxacin should be confined to "rescue" therapy only, in order to avoid a further rapid increase in *H. pylori* resistance to quinolone. Extending the duration of treatment to 14 days has been shown to improve eradication rates, but the impact of quinolone-containing *H. pylori* eradication on public health issues such as tuberculosis treatment in such a lengthy regimen is a concern. Exposure to quinolones is relevant to delays in the diagnosis of tuberculosis and the development of drug resistance. Tailored therapy to detect quinolone-resistant strains could offer better eradication rates. This can be achieved by using culture-based or molecular-based methods such as

real-time PCR to detect the presence of a *gyrA* mutation, which is predictive of treatment failure with triple therapy for quinolones, such as levofloxacin.

Conflict of Interests

The authors declare that there is no conflict of interest regarding the production of this paper.

Authors' Contribution

Seng-Kee Chuah and Wei-Chen Tai are co-first authors.

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Clinical Study

Levofloxacin-Amoxicillin/Clavulanate-Rabeprazole versus a Standard Seven-Day Triple Therapy for Eradication of *Helicobacter pylori* Infection

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The resistance rates of *Helicobacter pylori* to amoxicillin and metronidazole therapy are higher in eastern Taiwan as compared to national and worldwide rates. The high resistance rate in this territory justified a search for a better eradication regimen. We conducted an open-labeled, prospective, randomized, and controlled study in a tertiary referral hospital in eastern Taiwan. Between December 2007 and December 2009, a total of 153 *Helicobacter pylori*-positive, therapy-naïve patients with a positive rapid urease test were recruited for random assignment to two seven-day treatment groups: levofloxacin (500 mg), amoxicillin/clavulanate (875 mg/125 mg), and rabeprazole (20 mg) twice per day (LAcR) or clarithyromicin (500 mg), amoxicillin (1000 mg), and rabeprazole (20 mg) twice per day (CAR). *Helicobacter pylori* eradication was assessed using the ^{13}C-urea breath test or rapid urease test performed at least 4 weeks after the end of treatment. After exclusion, 146 patients were enrolled and allocated in the study. The *Helicobacter pylori* eradication rates analyzed by both intention to treat (78.1% versus 57.5%, $P = 0.008$) and perprotocol (80.9% versus 61.8%, $P = 0.014$) were significantly higher for the LAcR group. In conclusion, the seven-day LAcR regimen provided improved *Helicobacter pylori* eradication efficacy when compared with the standard CAR triple therapy in eastern Taiwan.

1. Introduction

Helicobacter pylori (*H. pylori*) colonizes the human stomach chronically and is the causative agent of numerous benign and malignant gastric diseases [1]. A seroprevalence study in Taiwan showed that the seropositivity of *H. pylori* infection was 54.4% [2]. Eradication of *H. pylori* with a standard triple therapy using a proton pump inhibitor (PPI), amoxicillin, and clarithromycin or metronidazole was recommended to prevent gastric cancer and to avoid recurrence of peptic ulcer diseases [1, 3]. The recommended duration of the standard triple therapy from Asia-Pacific Consensus Guidelines is

Table 1: Primary resistance rate of *H. pylori* reported in Taiwan (published since 2000 to 2010 D.C.)

Author (study period) [reference]	Location (region)	Metronidazole	Clarithromycin	Amoxicillin	Levofloxacin	Tetracycline
Hu et al. (2004~2005) [6]	Hualien (east)	51.9%	13.5%	36.1%	Nil	0%
Yang et al. (1997~1999) [7]	Taipei (north)	9% (6/67)	18% (12/67)	Nil	Nil	Nil
Poon et al. (1998~2000) [8]	Taichung (west)	41.7% (35/84)	10.7% (9/84)	0% (0/84)	Nil	Nil
Poon et al. (2001~2004) [8]	Taichung (west)	25.4% (34/134)	6.7% (9/134)	0% (0/134)	Nil	Nil
Hung et al. (1998~2007) [9]	Tainan (south)	27.6% (58/210)	9.5% (20/210)	1.0% (2/210)	5.7% (12/210)	0.5% (1/210)
Wu et al. (2007~2008) [10]	Kaohsiung (south)	33.5% (56/167)	6.6% (11/167)	0.6% (1/167)	10.2% (17/167)	0.6% (1/167)
Liou et al. (2007~2009) [11]	Taipei (north)	Nil	7.5% (20/266)	2.5% (7/279)	5.7% (16/280)	Nil

seven days [4]. Clarithromycin or metronidazole is suggested if the *H. pylori* local primary resistance rate is lower than 15~20% for the former or less than 40% for the latter [3]. Antimicrobial resistances in many countries have increased. As a consequence, the triple therapy eradication rate was less than 80% on an intention-to-treat (ITT) basis [5]. In eastern Taiwan, the primary resistance rates of metronidazole (51.9%), amoxicillin (36.1%), and clarithromycin (13.5%) in clinical isolates of *H. pylori* were higher [6] than those reported from other regions of Taiwan (Table 1) and worldwide [7–12]. However, the eradication rate of a seven-day triple therapy using clarithromycin and amoxicillin has never been reported for patients in eastern Taiwan.

Regimens containing levofloxacin (500 mg b.i.d.) plus amoxicillin (1 g b.i.d.) (LA) and a PPI have been evaluated recently as an alternative to the standard antibiotics [1, 13]. The use of LA-based regimens as a first-line treatment for *H. pylori* is encouraging but still controversial [11]. Beta-lactamase production in *H. pylori* is the principal mechanism of amoxicillin resistance [14]. In vitro studies [15, 16] and clinical trials [17–19] showed promising results by using amoxicillin plus a beta-lactamase inhibitor like clavulanic acid to attenuate *H. pylori* resistance to amoxicillin. Thus, our aim was to evaluate the efficacy and tolerability of a seven-day levofloxacin (500 mg b.i.d.), amoxicillin/clavulanate (1 g b.i.d.) plus rabeprazole (20 mg b.i.d) (LAcR) regimen versus the guideline-recommended seven-day triple therapy for treatment of *H. pylori* infection [4].

2. Methods

2.1. Study Population. In this single center prospective study, we included *H. pylori*-positive adult patients assessed by the rapid urease test between December 2007 and December 2009. We excluded patients under the age of 20, those who had received anti-*H. pylori* therapy previously, those with concomitant illness or conditions (i.e., cardiopulmonary, hepatic, renal, or neoplastic diseases), those who were pregnant or breast-feeding women, and those allergic to any of the drugs used. The protocol was approved by the Institutional Review Board (IRB) of Buddhist Tzu-Chi General Hospital (IRB 096-28) and registered in ClinicalTrials.gov (NCT01575899). Informed consents were obtained from all participants.

2.2. Study Design. Eligible patients were assigned into two groups by a computer generated random table with blocks based on gender. Sealed envelopes which were opened in the outpatient clinic without blinding were used for allocation concealment. The LAcR group received levofloxacin, 500 mg (Cravit, Daiichi-Sankyo, Japan) b.i.d., amoxicillin/clavulanate, 875 mg/125 mg (Augmentin, Glaxo-SmithKline, UK) b.i.d., and rabeprazole, 20 mg (Pariet, Eisai, Japan) b.i.d., for 7 days. The standard triple therapy group served as the control group and was treated with clarithyromicin, 500 mg (Klaricid, Abbott, USA) b.i.d., amoxicillin, 1000 mg (Amoxicillin capsule 250 mg, Yung-Shin, Taiwan) b.i.d., and rabeprazole, 20 mg b.i.d. (CAR), for seven days.

2.3. Drug Compliance and Adverse Events. Drug compliance was defined as intake of more than 80% of each prescribed medication. Compliance and incidence of adverse events were collected by phone calls and in outpatient clinics. Each symptom was graded as either absent or present. *H. pylori* eradication was established based on a negative ^{13}C-urea breath test or a negative rapid urease (CLO) test. The confirmation tests were carried out at least 4 weeks after completion of eradication therapy by operators unaware of the patients' medication and *H. pylori* status.

2.4. Statistical Analysis. The primary end point of this study was to evaluate the eradication rate of the LAcR regimen. The evaluation of *H. pylori* eradication efficacy was performed on both an intention-to-treat (ITT) and a perprotocol (PP) analyses. The sample size was predetermined for this paired cohort study, taking the following parameters into consideration: initial estimate of the difference in efficacy = 15% (i.e., 87% versus 72%) [5, 13]; alpha = 0.05; power = 80%, and gamma = 0.2. The number of patients thus calculated in each group was 88. The final sample size was 100 patients when assuming that 15% of the patients were lost to follow-up. Interim analyses with periodic reports every 4 months were requested by our IRB, which allowed investigators to adjust study course according to the interim results.

Categorical data were compared using the chi-square test employing Yates correction for continuity or Fisher's exact test as appropriate. Continuous data were compared with Student's t-test and expressed as mean ± SD. The stratified chi-square test was used for subgroup analysis between the

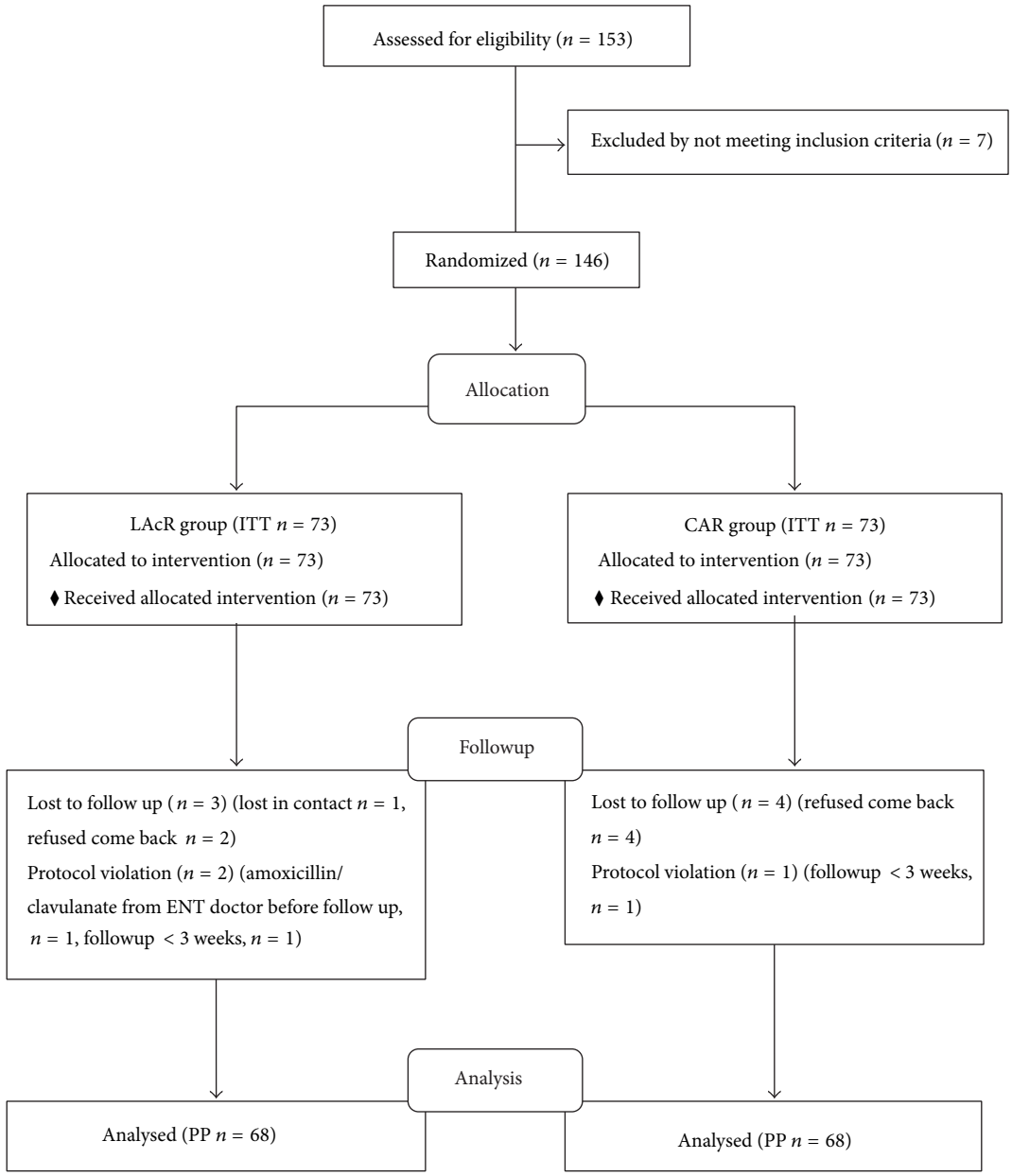

Figure 1: The participant flow chart.

two groups. Multiple logistic regression analyses were used for determination of major factors that affected treatment efficacy. Statistical analyses were performed using the SPSS 12.0 statistical software for Windows.

3. Results

3.1. Interim Analysis Result. The fifth interim analysis performed on December 2009 revealed that the difference in the eradication rates between the LAcR and CAR regimens was statistically significant ($P = 0.014$). According to the stopping boundaries corresponding to the fifth interim analysis for the Pocock (0.0158) or O'Brien-Fleming approach (0.0417) [20], this study was terminated early for ethical reasons. A total of 153 cases were screened, 7 cases were excluded, and 146 cases were enrolled and treated.

3.2. Patient Characteristics. The participant flow for this study is shown in Figure 1. As shown in Table 2, the baseline demographic data including age, gender, area of residence, endoscopic diagnosis, and follow-up methods were not significantly different between the two groups. The area of residence was defined by the domicile addresses registered by the participants. An address was considered "urban" if located in a city, while it was considered "rural" if in a township. Old population in this study, according to the current definition of the United Nations, was defined as ≥60 years old.

Table 2: Demographic characteristics of participants.

	All (n = 146)	LAcR (n = 73)	CAR (n = 73)	P value
Mean age (years)	53.73 ± 13.29	52.82 ± 12.08	54.63 ± 14.42	0.413
Age < 60 years old, n (%)	98 (67.1)	51 (69.9)	47 (64.4)	
Age ≧ 60 years old, n (%)	48 (32.9)	22 (30.1)	26 (35.6)	0.481
Gender				
Male, n (%)	71 (48.6)	38 (52.1)	33 (45.2)	
Female, n (%)	75 (51.4)	35 (47.9)	40 (54.8)	0.408
Resident area				
Urban, n (%)	54 (37.0)	30 (41.1)	24 (32.9)	
Rural, n (%)	92 (63.0)	43 (58.9)	49 (67.1)	0.304
Endoscopic finding				
With peptic ulcer, n (%)	65 (44.5)	30 (41.1)	35 (47.9)	
Without peptic ulcer, n (%)	81 (55.5)	43 (58.9)	38 (52.1)	0.405
Follow-up method				
C13 urea breath test	130 (89.0)	65 (89.0)	65 (89.0)	0.881
CLO test	9 (6.2)	5 (6.9)	4 (5.5)	
Lost to follow up	7 (4.8)	3 (4.1)	4 (5.5)	

Table 3: Comparison of eradication rate and subgroup analysis.

	Perprotocol analysis				Intention-to-treat analysis			
	LAcR, n (%)	CAR, n (%)	P value	Odd ratio (95% CI)	LAcR, n (%)	CAR, n (%)	P value	Odd ratio (95% CI)
All	55 (80.9)	42 (61.8)	0.014*	2.619 (1.20~5.70)	57 (78.1)	42 (57.5)	0.008*	2.629 (1.28~5.42)
Age								
<60 years old	37 (75.5)	31 (72.1)	0.710	1.194 (0.47~3.03)	37 (72.5)	31 (66.0)	0.479	1.364 (0.58~3.23)
≧60 years old	20 (95.2)	11 (42.3)	0.000*	27.273 (3.17~235)	20 (90.9)	11 (42.3)	0.000*	13.636 (2.62~70.91)
Gender								
Male	30 (81.1)	20 (64.5)	0.123	2.357 (0.78~7.11)	30 (78.9)	20 (60.6)	0.091	2.438 (0.86~6.94)
Female	25 (80.6)	22 (59.5)	0.060	2.841 (0.94~8.59)	27 (77.1)	22 (55.0)	0.044*	2.761 (1.01~7.55)
Resident area								
Urban	21 (77.8)	16 (76.2)	0.748	1.094 (0.28~4.23)	22 (73.3)	16 (66.7)	0.594	1.375 (0.43~4.44)
Rural	34 (82.9)	26 (55.3)	0.006*	3.923 (1.45~10.62)	35 (81.4)	26 (53.1)	0.004*	3.870 (1.50~10.02)
Endoscopic finding								
With peptic ulcer	22 (75.9)	22 (66.7)	0.426	1.571 (0.51~4.80)	22 (73.3)	22 (62.9)	0.368	1.625 (0.56~4.69)
Without peptic ulcer	33 (84.6)	20 (57.1)	0.009*	4.125 (1.38~12.36)	35 (81.4)	20 (52.6)	0.006*	3.938 (1.45~10.68)

*$P < 0.05$.

3.3. Effects of Therapy on H. pylori Eradication. The PP eradication rates (PP-ER) for the LAcR and the CAR groups were 55/68 (80.9%) and 42/68 (61.8%), respectively (P = 0.014). The ITT eradication rates (ITT-ER) for the LAcR and the CAR groups were 57/73 (78.1%) and 42/73 (57.5%), respectively (P = 0.008) (Table 3). In the subgroup analysis, compared with the CAR regimen, the LAcR therapy showed significantly higher PP-ER (52.6% versus 83.9%, P = 0.006) and ITT-ER (51.3% versus 77.1%, P = 0.021) in patients ≧54 years old, higher PP-ER (55.3% versus 82.9%, P = 0.006) and ITT-ER (53.1% versus 81.4%, P = 0.004) in those living in rural areas, and higher PP-ER (57.1% versus 84.6%, P = 0.009) and ITT-ER (52.6% versus 81.4%, P = 0.006) in those without endoscopic diagnosis of peptic ulcer diseases (Table 3). A

Table 4: Multiple logistic regression analysis.

	Perprotocol		Intention to treat	
	Odds ratio (95% CI)	*P* value	Odds ratio (95% CI)	*P* value
Age				
<60 years old	1.0 (referent)		1.0 (referent)	
≥60 years old	1.33 (0.61–2.92)	0.478	1.16 (0.55–2.47)	0.696
Gender				
Male	1.0 (referent)		1.0 (referent)	
Female	1.14 (0.53–2.46)	0.737	1.18 (0.57–2.44)	0.648
Resident area				
Urban	1.0 (referent)		1.0 (referent)	
Rural	1.49 (0.65–3.42)	0.342	1.12 (0.53–2.37)	0.775
Endoscopic finding				
With peptic ulcer	1.0 (referent)		1.0 (referent)	
Without peptic ulcer	1.05 (0.48–2.26)	0.909	1.05 (0.51–2.16)	0.900
Treatment				
CAR	1.0 (referent)		1.0 (referent)	
LAcR	2.67 (1.22–5.84)	0.014*	2.57 (1.24–5.33)	0.011*

*$P < 0.05$.

Table 5: Comparison of side effects.

	LAcR[a]	CAR	*P* value
All, *n* (%)	10 (13.7)	11 (15.1)	0.814
Abdominal pain	2	2	
Flatus/abdominal fullness	1	3	
Loose stool/diarrhea	3	1	
Nausea/hiccough	4	4	
Vomiting	2[a]	0	
Change in appetite	2	4	
Insomnia	1	0	

[a]One of the two cases stopped the LAcR therapy due to severe vomiting.

multivariate logistic regression analysis confirmed that the unique factor that led to successful *H. pylori* eradication was the modality of treatment (Table 4).

3.4. Tolerance to H. pylori Eradication Therapy. One patient from the LAcR group developed severe vomiting during the therapy. This patient received supportive treatment at the emergency room. After recovery, she stopped the LAcR regimen and was lost to follow-up. The association between this event and the LAcR therapy could not be confirmed. Mild adverse events were reported by a few patients in both groups without significant differences (Table 5).

4. Discussion

Our study results suggest that the standard seven-day triple therapy is not suitable for patients in Eastern Taiwan because the *H. pylori* ITT-ER was only 57.5%. The LAcR regimen achieved a significantly higher *H. pylori* eradication rate, suggesting that the use of amoxicillin/clavulanate can increase the overall response rate. In addition, the LAcR regimen may have attenuated the influence of some socioeconomic factors contributing to a high failure rate by the standard triple therapy among rural residents. In fact, we found that the differences on primary resistance rates of metronidazole (52.8% versus 47.7%, $P = 0.95$), amoxicillin (22.2% versus 25.0%, $P = 0.98$), and clarithromycin (13.9% versus 22.7%, $P = 0.58$) in clinical isolates of *H. pylori* from patient living in urban and rural areas of eastern Taiwan were not statistically significant [21].

The efficacy of this seven-day levofloxacin plus amoxicillin/clavulanate (LAc) based regimen has never been reported previously. However, we found a similar study in northern Taiwan that evaluated a seven-day regimen using LA as a first-line therapy. As expected, this "LA" combination revealed an ITT-ER of 74.2%, which is inferior to our "LAc" combination with an ITT-ER of 78.1% [11].

Two studies evaluated the antiresistance effect of amoxicillin/clavulanate to its counterpart, amoxicillin, in a seven-day PPI, clarithromycin plus amoxicillin/clavulanate or amoxicillin regimen. One (with omeprazole) showed a significant improvement in the ITT-ER (86.6% versus 66.6%, $P < 0.05$) [17]. The second study (with esomeprazole) showed a positive trend but did not achieve statistical significance (ITT-ER 72% versus 62%, $P = 0.2188$) [18]. Similarly, a trial comparing omeprazole, azithromycin plus amoxicillin/clavulanate, or amoxicillin also showed a beneficial trend (ITT-ER 86% versus 82%, $P = 0.801$; PP-ER 91.5% versus 85.4%, $P = 0.543$) but did not attain statistical significance [19]. In two studies, the omeprazole, metronidazole plus amoxicillin/clavulanate regimen for 2 weeks provided an 80.5% PP-ER in children from Iran [22] and a 76.4% PP-ER in adults from China [23].

Other strategies to increase the eradication rate when confronted with antibiotic resistance are to increase dosage and to extend treatment duration. Better *H. pylori* eradication rates have also been achieved elsewhere by 10- to 14-day

versus seven-day LA regimens [24–28] with only two studies having less than 80% ITT-ER [24, 29]. We hypothesize that if we extend the duration of the LAcR regimen to 10–14 days, we will anticipate an ITT-ER beyond 80%. The optimal dosage and duration of a LAc-based regimen for an area with high *H. pylori* resistance to amoxicillin and/or clarithromycin require more investigations.

In this study, we chose rabeprazole as the PPI because its metabolism is less affected by CYP2C19 [30, 31] and CYP3A4 genotypes [31]. As a result, we anticipated less interpersonal variation in drug response, but this strategy may lead to a possible reduction in the synergistic effect of a PPI with antibiotics like clarithromycin [32, 33], metronidazole [33], or fluoroquinolone [34]. A study in China reported an ITT-ER of 75.4% for a seven-day LA-10 mg rabeprazole regimen, which was inferior to those of a seven-day LA-20 mg esomeprazole (85.2%) and a seven-day LA-40 mg esomeprazole (87.1%) regimen, respectively [35]. Thus, the susceptibility variation of CYP2C19/CYP3A4 genotypes and types/dosage of various PPIs used in this LAc-based regimen are also important considerations to improve *H. pylori* eradication rate. We have been engaged in an investigation of the influence of CYP2C19 genotypes on the efficacy of *H. pylori* eradication by the LAcR regimen as a second-line treatment.

We found that the *H. pylori* eradication rate of this LAcR regimen was not affected by common host factors (i.e., old age, rural residence, and ulcer/nonulcer status) that could affect the standard triple therapy. The poorer *H. pylori* eradication rate by the standard triple therapy in rural area may not be true in different countries. For example, rural areas in northern Wales had a higher ITT-ER (92%) by the standard triple therapy [36] when compared to rural China (PP-ER 69.59%, ITT-ER 65.6%) [37]. Our study results first point out that *H. pylori* eradication rate could be affected by problems in rural areas such as poor sanitary conditions and personal hygiene, antibiotic abuse in agriculture, limited education resources, and inadequate access to clean water. Since the majority of previous clinical trials on *H. pylori* treatment were performed in large city hospitals, the influence of rural residence on *H. pylori* eradication rate needs more therapeutic trials to confirm. Above all, one of the advantages of the LAcR regimen for first-line use is that clinicians need not be concerned about patients' socioeconomic status before prescribing this regimen in an area with high *H. pylori* resistance rate to conventional antibiotics. Their concerns can focus on penicillin allergy, compliance, and possible side effects. The limitation of this study is that the local *H. pylori* resistance rates to levofloxacin and amoxicillin/clavulanate are unknown.

5. Conclusion

The seven-day LAcR regimen for *H. pylori* eradication is a viable alternative to the standard seven-day clarithromycin-based triple therapy for eastern Taiwan. It is a rescue regimen before the discovery of an optimal first-line therapy for a region with high ampicillin and/or clarithromycin resistance rates. The efficacy of the standard eradication therapy is probably reduced by the high *H. pylori* resistance rate to amoxicillin and/or clarithromycin.

Conflict of Interests

The authors declare that they have no conflict of interests regarding the publication of this paper.

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