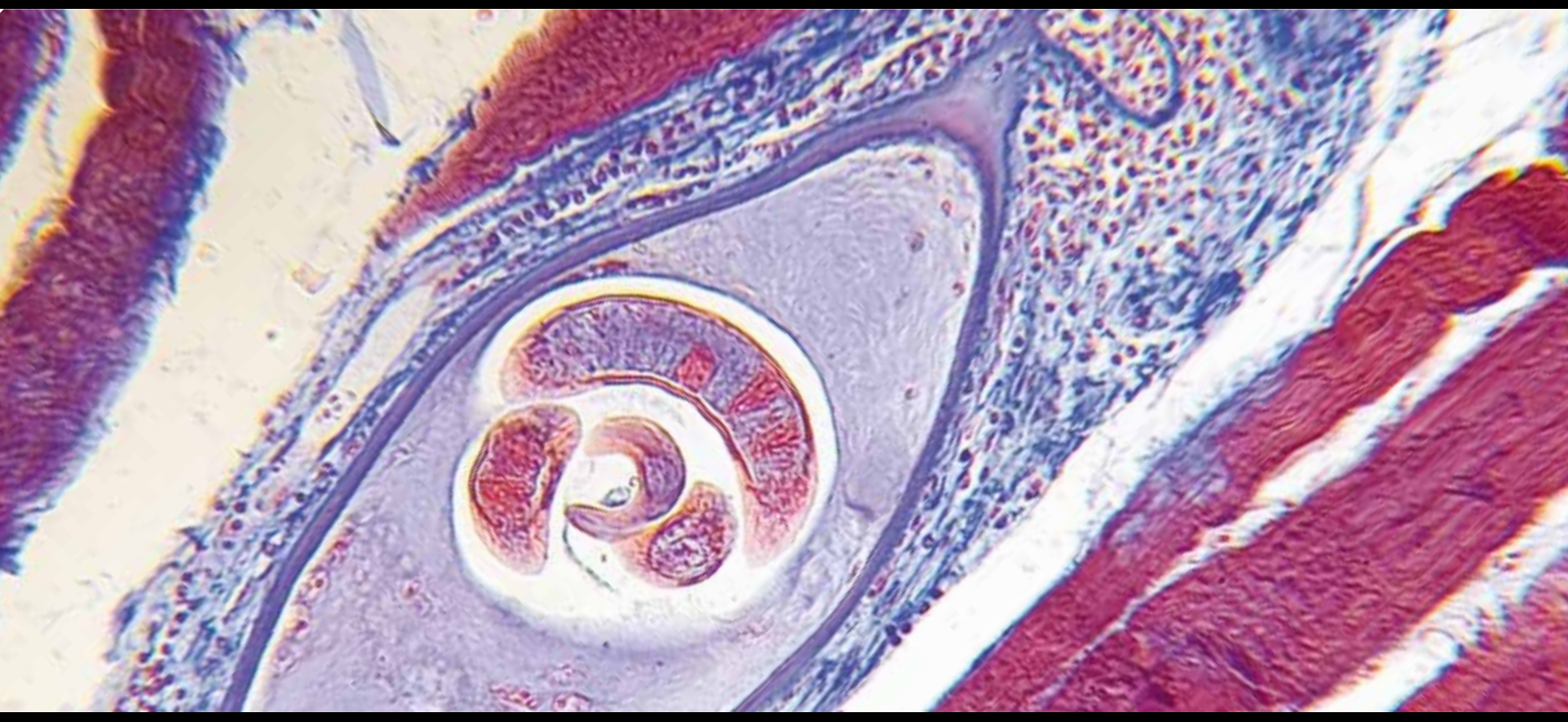
Gastroenterology Research and Practice

Helicobacter pylori Infection and Upper Gastrointestinal Disorders

Guest Editors: Vikram Kate, N. Ananthakrishnan, Frank I. Tovey, and N. K. Maroju



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Editorial Board

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Editorial

Helicobacter pylori Infection and Upper Gastrointestinal Disorders

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It has been thirty years since the two Australians Robin Warren and Barry Marshall discovered *Helicobacter pylori* (*H. pylori*) in 1983 [1]. In order to fulfil the Koch's postulates, Marshall and Morris drank a solution which was a suspension of *H. pylori*. This produced gastritis from which the bacteria could be reisolated [2]. It will be interesting to see how the approach to treatment has progressed in these three decades after the discovery of the organism-based both on consensus guidelines and other research in this field. The changes in the consensus statements have been highlighted. The isolation of *H. pylori* from the gastric mucosa and the report of the organism's urease activity generated excitement especially when it was postulated by Marshall that these microorganisms could be the cause of gastritis and could be a dominant etiological factor in the pathogenesis of peptic ulcer disease (PUD). With the isolation of *H. pylori*, floodgates opened to a new era of discovery and understanding of gastroduodenal pathology. These results were a paradigm shift from the earlier belief that PUD disease was related to stress, lifestyle, and acid secretion based on the dictum of Schwarz "no acid no ulcer." The early nineteen eighties when Warren and Marshall reported their findings coincided with omeprazole belonging to the group of proton pump inhibitors (PPIs) being introduced. This PPI was documented as a potent antisecretory agent which yielded very good results for ulcer healing and achieving a potential cure for patients with PUD when compared to the earlier drugs belonging to the group of H_2 receptor antagonists. Hence there was lot of scepticism in the gastroenterology community world over to accept that PUD was the result of infection. However, it was found that patients with PUD continued to have remission of the disease even after cessation of antisecretory therapy.

The knowledge about *H. pylori* continued to evolve at a rapid rate, and in 1994 the first guidelines on treatment of infection with this organism were published by the US National Institute of Health (NIH) in JAMA [3]. The consensus statement was that patients with PUD and *H. pylori* infection require eradication of *H. pylori* and antisecretory drugs whether on first presentation or on recurrence of the disease, whereas in patients with nonulcer dyspepsia (NUD) with *H. pylori* infection the value of treating *H. pylori* infection remained to be determined. It was also suggested in the consensus statement that the relationship between *H. pylori* infection and gastric cancers required further exploration.

The discrepancy in the treatment protocols in different countries and lack of national guidelines called for another meeting of The European *Helicobacter pylori* Study Group (EHPSG) at Maastricht, The Netherlands, in 1996, to form guidelines for general health care issues pertaining to *H. pylori* infection [4]. In addition to the recommendations of the NIH, treatment was strongly recommended for patients with low-grade gastric-mucosa-associated lymphoid tissue (MALT) lymphoma, bleeding PUD, gastritis with severe abnormalities, and following early resection for gastric cancer. It was also advised that eradication was advisable in patients less than 45 years of age with functional dyspepsia

in whom alarm symptoms were ruled out. The Maastricht guidelines advised noninvasive tests such as the urea breath test (UBT) or serology for diagnosis before therapy. Eradication was also advised in first degree relatives of gastric cancer patients, planned or existing (nonsteroidal anti-inflammatory drug) NSAID therapy, and following surgery for peptic ulcer. The seven-day standard triple therapy comprising PPI with two antibiotics was recommended in supersession of the previous classical bismuth triple therapy as it was found to be superior in efficacy, with fewer side effects and better compliance. An eradication rate of over 80% on intention-to-treat (ITT) basis was considered satisfactory.

In the next four years, significant progress was made in different aspects of *H. pylori*-associated disease which necessitated the second meeting of the EHPSG to update the previous guidelines [5]. This meeting of the experts was held again at Maastricht in the year 2000. All the strongly recommended indications for *H. pylori* eradication from the earlier Maastricht guidelines were reinforced in this meeting. In patients with PUD, it was recommended that this should include active and inactive disease, complicated disease, and also following surgery for peptic ulcer. The importance of eradication of *H. pylori* was stressed again in first degree patients with gastric cancer and patients with functional dyspepsia. The benefit in patients with functional dyspepsia was limited and was seen in less than 10% of patients; however, this was comparable to other treatments with antisecretory or antinociceptive drugs [6, 7]. It was stated that there was strong evidence that eradication of *H. pylori* is not associated with the development of gastroesophageal disease (GERD) in most cases nor does it exacerbate GERD [8, 9]. The guidelines stated that it was advisable to eradicate *H. pylori* in patients with GERD needing long-term profound acid suppression. The meeting also concluded that *H. pylori* and NSAID use are independent factors for PUD. *H. pylori* eradication was not indicated in extra-alimentary tract disorders. A modification on the Maastricht I report was that ranitidine bismuth citrate (RBC) combined with clarithromycin, amoxicillin, or metronidazole was included as first line triple therapy as it demonstrated similar efficacy to standard triple therapy where a PPI replaced RBC combined with two antibiotics [10]. A seven-day standard triple therapy was recommended. Quadruple therapy comprising a PPI, bismuth, metronidazole, and tetracycline was recommended as second-line therapy. Confirmation of eradication was advised at the end of 4 weeks following therapy by using non-invasive tests like UBT or stool antigen test (SAT) when endoscopy was not indicated for assessing the primary disorder. Serology was considered as an inappropriate method to determine eradication.

The Third Maastricht Consensus Conference convened to update guidelines on the management of *H. pylori* infection was held in Maastricht in 2005 [11]. The modified recommendations of this meeting included for the first time eradication of *H. pylori* in patients with extraintestinal diseases such as iron-deficiency anemia (IDA) and immune thrombocytopenic purpura (ITP) based on the reversal of IDA and significant positive platelet increase in patients with ITP [12, 13]. *H. pylori* was not found to have any proven role in other extraintestinal disorders. Eradication was recommended in naive NSAID users as it was likely to prevent peptic ulcer and bleeding. However, in patients receiving long-term NSAIDs with peptic ulcer or peptic ulcer bleeding, PPI maintenance treatment was considered superior to *H. pylori* eradication therapy in preventing ulcer recurrence or bleeding. Contrary to previous opinion it was found that there is a negative association between the prevalence of *H. pylori* and GERD especially with CagA positive strains as the incidence of Barrett's esophagus and gastroesophageal (GE) was reported to be lower in them [14]. However, it was reemphasized that eradication of *H. pylori* does not exacerbate GERD either when left untreated or when patients are being treated with PPI for GERD [11]. The duration for standard triple therapy was increased to 14 days. This was considered superior to seven-day therapy unless local studies showed that seven-day therapy was effective [15]. In areas with low metronidazole resistance (<40%), the combination of PPI-clarithromycin-metronidazole was superior to PPI-clarithromycin-amoxicillin independent of the sensitivity to clarithromycin [16]. SAT using monoclonal antibodies was preferable to polyclonal antibody test. A laboratory-based test was superior to office-based test.

The most recent fourth conference in this series was held in Florence in 2010. However, the Maastricht methodology was used for updating of the guidelines for *H. pylori* treatment. Hence it was termed as the Maastricht/Florence consensus report [17]. It was mentioned that patients who are on long-term PPIs with *H. pylori* infection are associated with corpus-predominant gastritis which can progress to atrophic gastritis. In these patients, eradication of *H. pylori* heals the gastritis and prevents progression to atrophic gastritis [18]. Apart from IDA and ITP, the other extraintestinal disorder in which eradication of *H. pylori* was recommended was Vitamin B12 deficiency. It was reinforced that SATs by a laboratory method using monoclonal antibodies should be used as an alternative to UBT as a non-invasive method to detect eradication of *H. pylori* [19, 20]. In areas of high clarithromycin resistance (more than 15–20%), bismuth quadruple therapy was recommended. If bismuth-based therapy was not available, then nonbismuth quadruple therapy or sequential therapy was indicated [21, 22]. *H. pylori* eradication to prevent gastric cancer was recommended in populations at high risk. This was in contradistinction to earlier guidelines which recommended eradication only after gastrectomy for gastric surgery. Eradication was also recommended in patients with risk of severe pan-gastritis, corpus predominant gastritis, or severe atrophy. Patients with strong environmental risk factors like heavy smokers or who have high exposure to dust, coal, cement, or working in quarries should have therapy for *H. pylori*.

It is interesting to see the gradual change in the recommendations for diagnosis and treatment of *H. pylori* infection over these years and the appearance of extraintestinal diseases as an indication for eradication. However, more and more diseases continue to be attributed to infection with this organism or are shown to be associated with infection with this organism such as inflammatory bowel disease or colonic carcinoma. We will have to wait till future studies throw some

light on the causal association of this organism with other extra-alimentary diseases.

Vikram Kate
Nanda K. Maroju
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Review Article

Helicobacter pylori-Induced Chronic Gastritis and Assessing Risks for Gastric Cancer

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Chronic gastritis is an inflammation of the gastric mucosa and has multiple etiologies. Here we discuss the pathological alterations induced by *Helicobacter pylori* (HP) leading to chronic gastritis and the epigenetic bases underlying these changes. We review the histology of the normal gastric mucosa and overview the role of HP in the multistep cascade of GC. We attempt to define the role of the Operative Link for Gastritis Assessment (OLGA) staging system in assessing the risk of GC. The epigenetic bases of chronic gastritis, mainly DNA methylation, are presented through examples such as (i) the methylation of the promoter region of E-cadherin in HP-induced chronic gastritis and its reversion after HP eradication and (ii) the association of methylation of the promoter region of Reprimo, a p53-mediated cell cycle arrest gene, with aggressive HP strains in high risk areas for GC. In addition, we discuss the finding of RPRM as a circulating cell-free DNA, offering the opportunity for noninvasive risk assessment of GC. Finally, the integration of OLGA and tissue biomarkers, by systems pathology approach, suggests that severe atrophy has a greater risk for GC development if, in addition, overexpressed p73. This trial is registered with ClinicalTrials.gov NCT01774266.

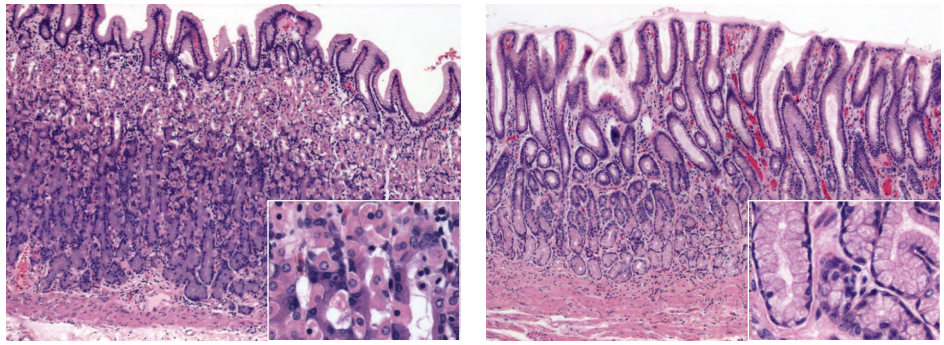
1. Introduction

Since 1870, both human and veterinary pathologists have described bacterial infections based on the observation of tiny curved bacteria within gastric mucosa [1, 2]. However, these organisms were dismissed as irrelevant contaminants. In 1947, when gastroscopy was first being used, Schindler deemed gastritis as "one of the most debated diseases of the human body" and predicted that its significance would be discussed "for some time to come" [3]. Schindler himself claimed that the "bacteriological etiology of chronic gastritis has not been convincingly proved in a single case" [3]. In 1984, Marshall and Warren proposed that chronic "idiopathic" gastritis had a bacterial cause, that is, *Helicobacter pylori* [4]. Their hypothesis was met with great skepticism. However, within a few years, the association between *H. pylori* gastritis, peptic ulcer, and gastric cancer came to be acknowledged and ultimately accepted [4]. For the purpose of this paper, we will focus mainly on the cascade of events produced by *Helicobacter pylori* infection leading to chronic changes in the gastric mucosa and the risk assessment for the development of gastric cancer. In addition, we will explore the epigenetic bases that underlie the changes of chronic gastritis.

2. Normal Gastric Histology

In order to recognize pathologic tissue responses in gastritis, it is essential to know the spectrum of normal gastric mucosa histology patterns. Normal gastric mucosa is formed by the epithelial/glandular and lamina propria components. The epithelial component consists of the foveolar epithelium, which is formed by tall columnar mucous cells with basally situated nuclei and supranuclear collections of closely packed small mucus globules that discharge their content onto the surface, forming an adherent protective lubricant layer that lines the lumen. The glandular component changes depending on its location in the stomach.

(1) Cardiac glands are limited to a narrow region of the stomach (the cardia) that surrounds the esophageal



(a) (b)

Figure 1: Normal gastric mucosa histology. ((a); H&E 10x; square 40x) Fundic glands are simple, branched tubular glands that extend from the bottom of the gastric pits to the muscularis mucosae; the more distinctive cells are parietal cells. ((b); H&E 10x; square 40x) Antral mucosa is formed by branched coiled tubular glands lined by secretory cells similar in appearance to the surface mucus cells.

orifice. They are tubular, somewhat tortuous, and occasionally branched, and are mainly formed by mucus-secreting cells, with occasional interspersed enteroendocrin cells.

(2) Fundic glands are present throughout the entire gastric mucosa, except for relative small regions occupied by cardiac and antral-pyloric glands. The fundic glands are simple, branched tubular glands that extend from the bottom of the gastric pits to the muscularis mucosae (Figure 1), and are formed by four functional types of cells: mucous neck cells, chief cells, enteroendocrine cells, parietal cells (also called oxyntic cells), and undifferentiated cells.

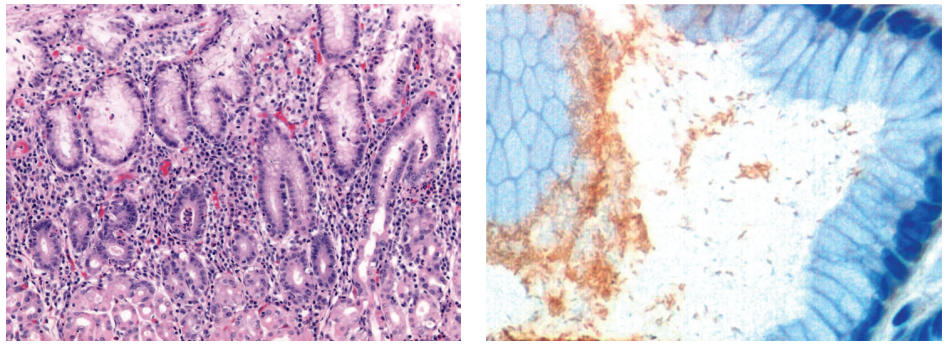
(3) Antral-pyloric glands are located in the *pylori* antrum (the part of the stomach between the fundus and the pylorus). They are branched coiled tubular glands and are lined by secretory cells similar in appearance to the surface mucus cells (Figure 1), suggesting a relative viscous secretion. Enteroendocrine cells are found interspersed within the gland epithelium along with occasional parietal cells.

The lamina propria is relatively scant and restricted to the limited spaces surrounding the gastric pits and glands. The stroma is composed of reticular fibers with associated fibroblasts and smooth muscle cells. It is also composed of lymphocytes, plasma cells, macrophages, and some eosinophils. The lymphocytes are predominantly immunoglobulin (Ig) A-producing B cells. IgG- and IgM-secreting cells are also present. Under normal conditions, intraepithelial lymphocytes are not present anywhere in the gastric mucosa. There is also a small number of lamina propria T cells, neutrophils, and mast cells. The lamina propria also contains capillaries, arterioles, and nonmyelinated nerve fibers. Small lymphoid aggregates, usually located in close proximity to the muscularis mucosae at the base of the lamina propria, especially at the corpus, could be present in normal gastric mucosa. In contrast, the presence of lymphoid aggregates with germinal centers is extremely rare in the mucosa of normal *H. pylori* negative adults [5].

3. Histopathology of *Helicobacter pylori*-Induced Chronic Gastritis

H. pylori is a microaerophilic gram-negative bacteria. The early phase of *H. pylori* infection elicits an acute inflammatory response that is either asymptomatic, or symptomatic with short-lived clinical manifestations such as nausea and vomiting, that evolve to a long-standing chronic gastritis [4]. Its prevalence goes from less than 15% in some populations to virtually 100%, depending on socioeconomic status and country development [6]. In industrialized countries (Western Europe, United States, Canada, and Australia), exposure tends to occur later in life, which results in a lower percentage of infected adults. An average of 20% to 30% of adults is infected by age 50 [6] and the prevalence of *H. pylori* infection has been steadily declining in emerging countries, which is probably a reflection of improved sanitary conditions, as well as the widespread use of antibiotics. The foveolar epithelium produces a thick layer of mucus that plays a protective role. This mucus layer is the primary site for *H. pylori* colonization [7]; so, they characteristically attach to the surface mucous cells, but do not penetrate them. In chronic infection, *H. pylori* contacts the surface epithelial membrane, producing prominent epithelial degeneration [8, 9]. The cells often become irregular and cuboidal in shape, showing a decrease in apical mucin content, as well as occasional "drop outs," which leaves small gaps in the epithelium and contributes to a ragged, disorderly appearance.

H. pylori preferentially colonize the antrum, but they may infect any part of the stomach where it causes gastritis. When treated, the bacteria migrate from the antrum to the corpus, decreasing the activity of antral gastritis. Marked neutrophilic infiltrates appear in the mucous neck region and lamina propria in early acute gastritis (Figure 2); when severe, they aggregate in the pit lumens to form pit abscesses. Both the neutrophils and the *H. pylori* destroy the epithelium, causing the mucous neck cells to proliferate in an effort to replace the dying cells. The regenerative pit bases are characterized by mucin loss, cytoplasmic basophilia, increased mitoses, and hyperchromatic nuclei that are sometimes severe enough to mimic dysplasia.



(a) (b)

Figure 2: Early acute superficial gastritis. ((a); H&E 20x) Marked neutrophilic infiltrates appear in the mucous neck region and lamina with a pit micoabscess. ((b); *H. pylori* immunostaining: rabbit polyclonal; clone CH-20 429, Novocastra; 40x) Numerous *H. pylori* bacteria are present in the superficial foveolar epithelium.

Neutrophilic inflammation and the presence of lymphoid follicles with germinal centers are the two most distinctive histological features of *H. pylori* infection, and its eradication causes rapid neutrophil disappearance; thus their continued presence is considered a valuable indicator of therapeutic failure. The surface changes reverse rapidly, and the epithelial cells acquire their normal shape and spatial organization within a few days of *H. pylori* eradication. However, any atrophy that had developed remains, as did the lymphoid aggregates [10]. These features become permanent components of the once-infected gastric mucosa.

4. Grading the *Helicobacter pylori*-Induced Chronic Gastritis: The Updated Sydney System

The most widely used grading system for gastritis is the Updated Sydney System [11]. The system provides guidelines for generating systematic and uniform diagnostic reports. The goal of the Sydney System is to make gastric biopsy pathology reporting consistent, so that clinical studies can be performed and evaluated in a meaningful manner. The system classifies chronic gastritis on the basis of topography, morphology, and, when possible, etiology, into three broad categories: acute, chronic, and special (or distinctive). The biopsy protocol recommends that specimens from three compartments (i.e., antrum, incisura angularis, and corpus) should be separately designated when submitted to the pathology laboratory. Each relevant pathologic feature (density of *H. pylori*, intensity of neutrophilic and mononuclear inflammation, atrophy of the antrum and corpus, and intestinal metaplasia) should be graded on a standardized visual analogue scale (Figure 3). Each feature is assigned either a numeric or descriptive value: 0 for absent, 1 for mild, 2 for moderate, and 3 for marked (or severe). The values of each specimen are then averaged separately for each anatomic compartment (antrum and corpus). The next step is to document the degree of inflammation in the two main gastric compartments (antrum and corpus) and to determine whether the inflammation is similar in intensity (i.e., pangastritis) or more severe in either the antrum (antrum-predominant gastritis) or the corpus (corpus-predominant gastritis).

5. *Helicobacter pylori*-Induced Chronic Gastritis and Multistep Cascade of Gastric Carcinogenesis

In gastric cancer development, *H. pylori*-induced chronic gastritis is the first step of the so-called multistep cascade of gastric cancer. The cascade sequence of gastric carcinogenesis includes the nonatrophic chronic gastritis, multifocal atrophic gastritis, intestinal metaplasia, low-grade dysplasia (low-grade noninvasive neoplasia), high-grade dysplasia (high-grade noninvasive neoplasia), and invasive adenocarcinoma as described by Correa as the "human model of gastric carcinogenesis" [12]. This multistep model hypothesizes that the sequence of lesions reflects a dynamic process from a naïve inflammation caused by *H. pylori* infection to a fully malignant neoplasm of the stomach [13–16]. Independent epidemiological studies have confirmed that these entities are all linked through a sequential cause-effect relationship, thus supporting the concept of a human model for gastric carcinogenesis [17–19]. In a recent review and update of this model, it is also postulated that *H. pylori* is present not only in the first step of gastric mucosa inflammation but as an etiological factor in every step of the precancerous cascade [20]. In the first step, *H. pylori* infection targets normal mucosa with well-preserved gastric glands, by definition such gastritis is nonatrophic. At this point, it can be cured by clearing *H. pylori* infection or it may be evolving in two ways: it can remain as nonatrophic or it progresses in severity, leading to damage to the gastric glands, which may eventually disappear [20]. The progression depends on the interplay of three sets of etiological factors: infectious agent, host's genetic susceptibility and external environment; these determine the susceptibility and severity of outcome in the subset of individuals that develop clinical disease [20, 21]. The presence of virulent factors in the infecting *H. pylori* strain is a known determinant factor of the outcome of the infection. Infection

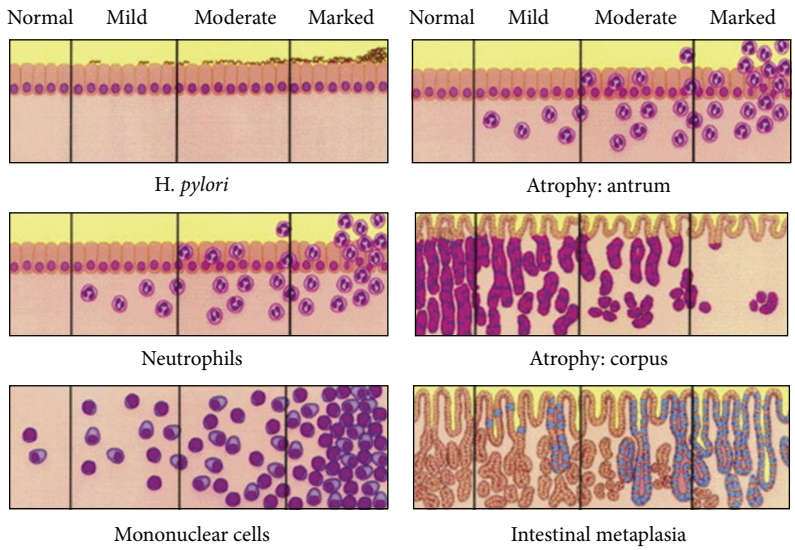


Figure 3: The Updated Sydney System visual standardized visual analogue scale. Each feature is assigned either a numeric or descriptive value: 0 for absent, 1 for mild, 2 for moderate, and 3 for marked (or severe). Taken from Dixon et al. [11].

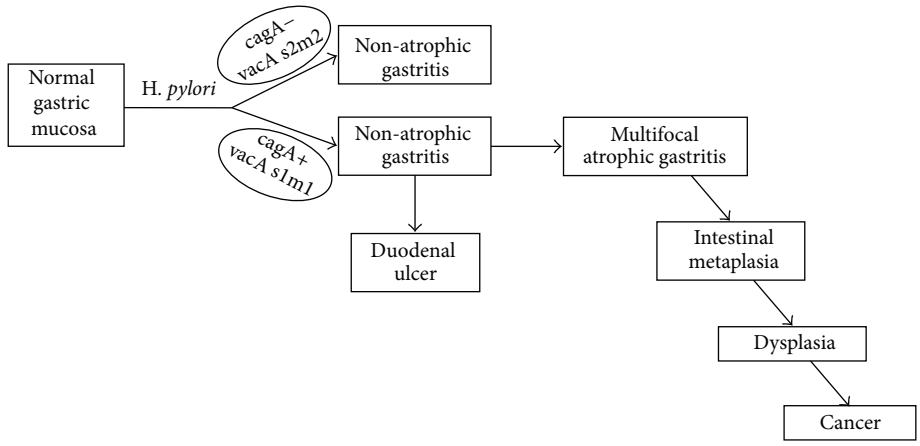
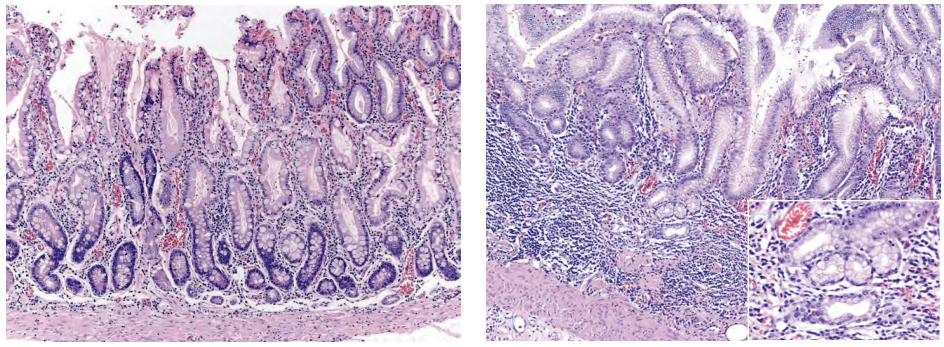


Figure 4: Multistep cascade of gastric cancer. This sequence begins with the infection of *H. pylori* to sequential steps of the precancerous cascades. Taken from Correa and Piazuelo [20].

with cag-positive vacA s1m1 strains is associated with the development of gastric cancer, while cag-negative vacA s2m2 infection does not increase the risk of cancer and is associated to the persistency of nonatrophic gastritis (Figure 4) [20, 22].

6. Assessing Risks for Gastric Cancer: The Operative Link for Gastritis Assessment (OLGA)

The risk of malignant transformation of the lesions associated with the multistep cascade of gastric cancer is poorly defined. Long-term follow-up studies have shown a risk from 10% to 17% in the case of dysplasia [23–27]. For intestinal metaplasia, the risk assessment has conflicting results and therefore a limited clinical value [28–32]. The recently developed Operative Link for Gastritis Assessment (OLGA) staging system [33], through the evaluation of the extension and site of the atrophic changes, is an attempt to evaluate the risk of chronic gastritis to progress to intestinal metaplasia and gastric cancer [34–36]. Long standing *H. pylori* infection may lead to the loss of functional glands (atrophy) and replacement of the normal gland and foveolar epithelium by intestinal type cells (intestinal metaplasia) (Figure 5), the two main histological abnormalities invariable present as the background of gastric cancer [37]. The extent and site of the atrophic changes significantly correlate with cancer risk [38]. Two main types of atrophy can be recognized: one characterized by the loss of glands, accompanied by fibrosis or fibromuscular proliferation in the lamina propria, and the other characterized by the replacement of the normal (native) glands with metaplastic glands (i.e., glands that do not normally belong to that area) [39]. The degree of atrophy and metaplasia can be assessed with OLGA staging system for atrophy risk assessment [33]. This new system requires that specimens are taken according to the biopsy protocol



(a) (b)

Figure 5: Atrophy is the loss of appropriate glands. ((a); H&E 10x) Antral gastric mucosa with accentuated atrophy because replacement by extensive intestinal metaplasia. ((b); H&E 10x; square 20x) Fundic-corporal gastric mucosa with extensive loss of gastric glands, partially replaced by pseudo-pyloric metaplasia.

Table 1: The OLGA staging frame. Atrophy is scored as the percentage of atrophic glands and scored on a four-tiered scale. No atrophy (0%) = score 0; mild atrophy (1–30%) = score 1; moderate atrophy (31–60%) = score 2; 9 severe atrophy (>60%) = score 3. These scores (0–3) are used in the OLGA staging assessment in each 10 compartment. Taken from Rugge et al., Dig Liver Dis 2011; 43:S373-84 with permission of Elsevier.

		Corpus			
	Atrophy score	No atrophy (score 0)	Mild atrophy (score 1)	Moderate atrophy (score 2)	Severe atrophy (score 3)
Antrum	No atrophy (score 0) (including incisura angularis)	Stage 0	Stage I	Stage II	Stage II
	Mild atrophy (score 1) (including incisura angularis)	Stage I	Stage I	Stage II	Stage III
	Moderate atrophy (score 2) (including incisura angularis)	Stage II	Stage II	Stage III	Stage IV
	Severe atrophy (score 3) (including incisura angularis)	Stage III	Stage III	Stage IV	Stage IV

of the Sydney System, and that atrophy is scored in a four-tiered scale (0–3) according to the visual analogue scale of the Houston-updated Sydney system. The stage resulting from the combination of atrophic changes was assessed in the two mucosal compartments considered herein (Table 1).

7. Assessing Risks for Gastric Cancer: The Epigenetic Bases of Chronic Gastritis

The molecular bases of the multistep process of gastric carcinogenesis are highly relevant since it contributes greatly to assess risks of gastric cancer. Therefore, chronic gastritis should be understood as a disturbance in the balance between tumor suppressor genes and oncogenes. Many tumor suppressor genes have been identified in gastric cancer as well as chronic gastritis [40]. For example, inactivation of p53 tumor suppressor gene, E-cadherin, and DNA mismatch repair genes (hMSH2 and hMLH1) responsible for loss of heterozygosity (LOH) and microsatellite instability (MSI) are well-recognized examples. However, multiple studies have shown that mutation and/or deletion is an infrequent mechanism of inactivating these well-established tumor suppressor genes [41]. In this scenario, epigenetic alterations, such as DNA methylation, have been proposed as an alternative mechanism for inactivation of tumor suppressor genes [41]. DNA methylation is a process in which cytosines acquire a methyl group in $5'$ position only if they are followed by a guanine (CpG site) [42]. Since DNA methylation has been considered as an excellent candidate to explain how environmental factors may increase the risk of cancer, it has been proposed as a key element for the early events of gastric carcinogenesis [43]. For example, Chan et al. [44] found that DNA methylation of the promoter region of E-cadherin has been associated with *H. pylori* infection. This association was independent of the age and/or type of gastritis [44]. Furthermore, the same authors [45] also demonstrate that *H. pylori* eradication with antibiotics reverses the DNA methylation of E-cadherin (Figure 6). Similarly, Maekita et al. [46] analyzed the effect of *H. pylori* infection on DNA methylation for multiple genes (HAND1, HRASLS, LOX, p16, P41ARC, and THBD) in *H. pylori* negative and positive healthy donors and gastric cancer patients. Among healthy donors, methylation levels were higher in *H. pylori* positives than in *H. pylori* negatives [46]. Taken together these data suggest that *H. pylori* infection induces DNA methylation mostly in premalignant conditions rather than gastric cancer itself [46]. Among other tumor suppressor genes inactivated

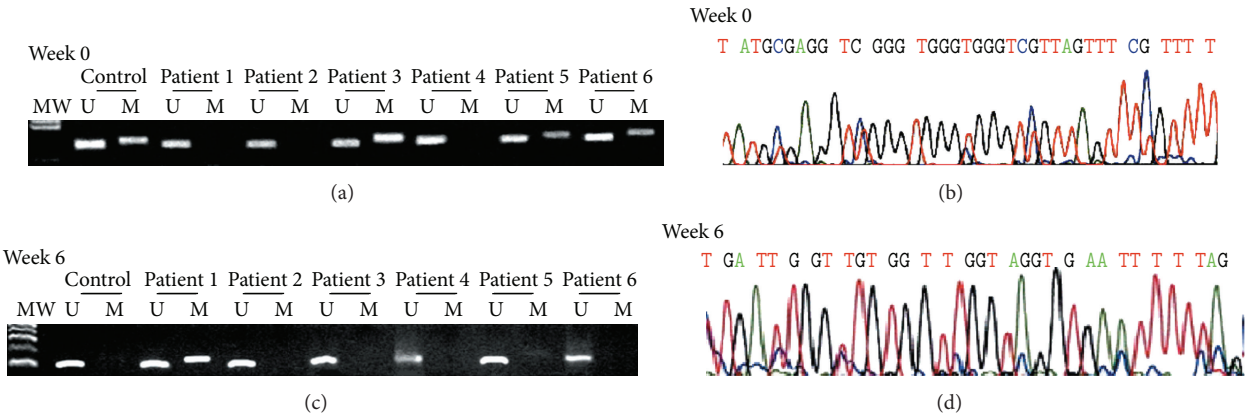


Figure 6: CpG island methylation pattern at the E-cadherin gene in gastric mucosa from patients with dyspepsia. (a) Before eradication of *H. pylori* (week 0), methylation was present in patients 3, 5, and 6. (b) The methylated product was confirmed by sequencing using the same methylated primer. (c) After eradication of *H. pylori* (week 6), methylation was not present in any patient. (d) The methylated product was again confirmed by sequencing using the same methylated primer. No methylated cytosine was seen. MW: molecular weight marker, U: unmethylated band, M: methylated band, red color: unmethylated cytosines converted to thymidine, blue color: methylated cytosines. Taken from Chan and Rashid [45].

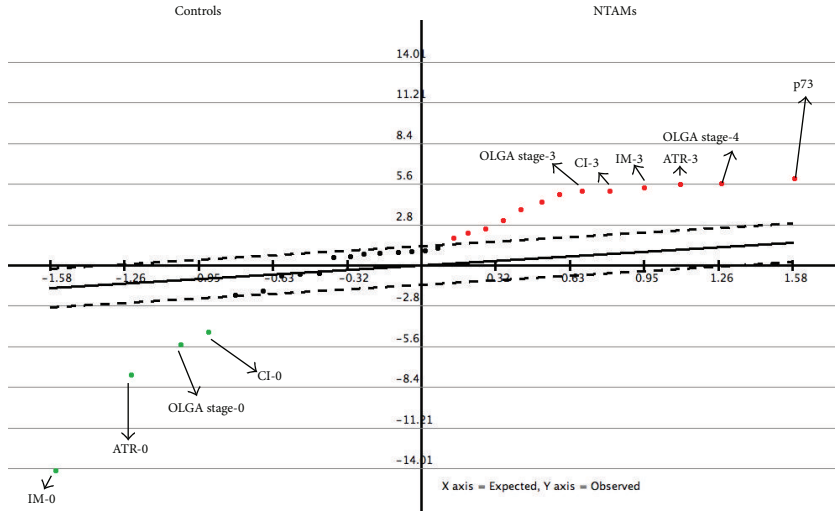


Figure 7: Serial analysis for microarray from nontumor adjacent mucosa (NTAM) and chronic gastritis controls. NTAM group is significantly characterized by the overexpression of p73, OLGA stages III to IV, and severe atrophy (ATR-3), intestinal metaplasia (IM-3), and chronic inflammation (CI-3) according to the Sydney System. Control group cases were significantly characterized by lack of intestinal metaplasia (IM-0), atrophy (ATR-0), and chronic inflammation (CI-0). False discovery rate = 0. Taken from Carrasco et al., [51].

by DNA methylation, Reprimo (RPRM), a downstream mediator of p53-induced G2 cell cycle arrest [47], has been recently associated with more aggressive *H. pylori* strains (cag-positive, vacA s1 m1, and EPIYA polymorphisms) in Colombian residents from areas with high incidence of gastric cancer [48]. Furthermore, we have identified that DNA methylation of RPRM is not only found in gastric mucosa but also in the plasma of gastric cancer patients [49]. Therefore, this circulating cell-free DNA offers the opportunity for noninvasive assessing risks for gastric cancer. Since a recent meta-analysis suggests that among several candidates, circulating cell-free DNA of RPRM methylation is the most promising [50], this issue is currently under evaluation (Detection of Methylated Reprimo in Plasma for Asymptomatic Gastric Cancer, DEMRAC study. Finally, we have recently attempted to identify specific tissue biomarkers for assessing the risk of premalignant gastritis [51]. To this purpose, we evaluate the tissue overexpression, as a consequence of DNA hypomethylation, of several oncoproteins associated with gastric cancer including STAT1, p73, FHIT, p16INK4a, BRCA1, HSP90, and EGFR. These tissue biomarkers were compared with *H. pylori* and the OLGA staging system. As expected, severe atrophy and OLGA stage IV were the most relevant histological features of premalignant gastritis. Among tissue biomarkers, overexpression of p73 was the most relevant finding. Both data were integrated by systems pathology approach by performing Significance Analysis of Microarrays [52, 53]. This approach shows that p73 is stronger as a single variable when compared with OLGA stage IV (Figure 7). Therefore, we believe that gastritis

with severe atrophy has a greater risk for developing gastric cancer if, in addition, overexpress p73 [51].

In summary the role of *H. pylori* in the development of chronic gastritis is not only associated to the bacteria itself but also to host and environmental factors. Assessing risks for gastric cancer can be achieved by the evaluation of clinical, morphological, and molecular factors. Among morphological criteria, atrophy and intestinal metaplasia can be evaluated via Sydney and OLGA approaches. Molecular factors that should be considered are mostly E-cadherin methylation, circulating cell-free DNA of RPRM methylation, and overexpression of p73. Finally, a system pathology approach allows integrating all these factors that might be useful in switching from an interpretive and subjective morphologically oriented approach to a more objective, evidence-based tissue marker approach.

Acknowledgment

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Clinical Study

Gallstones and Concomitant Gastric *Helicobacter pylori* Infection

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Background. The association of gallstones with *Helicobacter pylori* has been investigated but not clearly demonstrated. In this study, the presence of *H. pylori* in the gallbladder mucosa of patients with symptomatic gallstones was investigated. *Method*. Ninety-four consecutive patients with symptomatic gallstone disease were enrolled for the study. Gastroscopy and gastric *H. pylori* urease test were done before cholecystectomy to all patients who accepted. After cholecystectomy, the gallbladder tissue was investigated in terms of *H. pylori* by urease test, Giemsa, and immunohistochemical stain. *Results*. Overall 35 patients (37%) gallbladder mucosa tested positive for *H. pylori* with any of the three tests. Correlation of the three tests Giemsa, IHC, and rapid urease test was significant (r_s: 0590, $P > 0.001$). Rapid urease test was positive in the gastric mucosa in 47 (58.7%) patients, and it was positive in the gallbladder mucosa in 21 patients (22%). In 15 patients both gastric and gallbladder tested positive with the urease test. There was significant correlation of rapid urease test in both of gallbladder and gastric mucosa ($P = 0.0001$). *Conclusion*. Study demonstrates the presence of *H. pylori* in the gallbladders of 37% of patients with symptomatic gallstones.

1. Introduction

Gallstone disease is one of the most common problems affecting the digestive tract where autopsy reports show a prevalence of 11–36% [1]. The prevalence of gallstones is related to many factors including age, gender, and ethnic background. Women are three times more likely to develop gallstones than men, and first-degree relatives have a twofold increased prevalence [2]. However, the etiology of gallstone formation, beginning with a change in the composition of bile, leading to stones, is not clear.

The association between *Helicobacter pylori* (*H. pylori*) and gallstones has been investigated but not clearly demonstrated. *H. pylori* is a Gram negative and microaerophilic microorganism that can cause chronic gastritis, gastric and duodenal ulcers, gastric and pancreatic adenocarcinoma, and lymphoma of gastric mucosa-related lymphoid tissue (MALToma) [3–9]. The relationship of *H. pylori* with diseases of organs other than the stomach and duodenum has also been investigated and reported [10, 11]. Antibodies to *H. hepaticus*, often cross reacting with *H. pylori*, were detected in patients with chronic liver diseases [12]. Also, *H. pylori* have been detected in the gallbladder mucosa of patients with gallstones [13].

In this study, the presence of *H. pylori* in the gallbladder mucosa of patients with symptomatic gallstones undergoing cholecystectomy was investigated. Concomitant *H. pylori* infections of the gastric mucosa were also investigated to study the relationship of gastric *H. pylori* infections to gallstones. It was hypothesized that *H. pylori* infection of the gastric mucosa may have a role in the formation of gallstones.

2. Material and Methods

The study was conducted on patients undergoing laparoscopic cholecystectomy for symptomatic cholelithiasis in Marmara University Hospital and Maltepe University Hospital, Istanbul, Turkey. The Research Ethics Committee approved

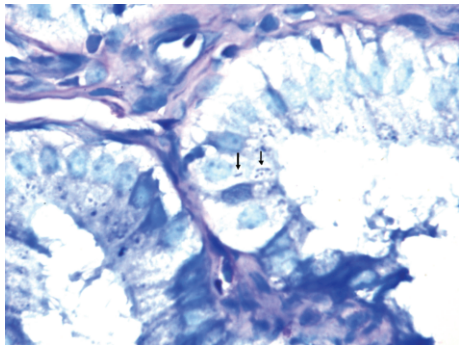

Figure 1: *H. pylori* within the gallbladder mucosa epithelium (MGG, ×1000).

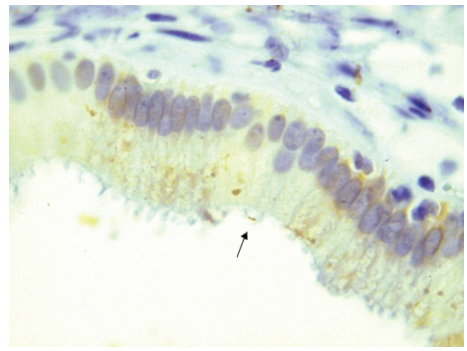

Figure 2: Immunopositivity for *H. pylori* along with the gallbladder mucosal columnar epithelium. Note that the bacilli are noninvasive and placed on the apical surface of the epithelium (×400).

the study (approval number: B.30.2.MAR.0.01.02/AEK/73), and all patients signed the informed consent.

2.1. Study Group. Ninety-four consecutive patients (31 male, 63 female; mean age 48) with symptomatic gallstone disease were enrolled for the study. Patients with acute cholecystitis, cholangitis, biliary and hepatic tumors, Crohn's disease, and previous gastric surgery were not considered suitable for evaluation. Patients undergoing ERCP (endoscopic retrograde cholangiopancreatography) and patients who had received *H. pylori* eradication treatment in the last 6 months were also excluded from the study. Gastroscopy and the gastric *H. pylori* urease test (Pronto Dry) were done before the surgical procedure for all patients who accepted.

2.2. Determination of H. pylori Status. After laparoscopic cholecystectomy, the gallbladder was opened in the operating room, and three strips of tissue were obtained from the infundibulum of the gallbladder. One sample was used for the rapid urease test (Pronto Dry), the second for aerobic and anaerobic culture, and the third for histopathologic evaluation. Gallstones were classified as cholesterol, pigment, and mixed stones based on their color and consistency [14, 15].

In those patients who had accepted to undergo gastroscopy prior to their laparoscopic cholecystectomy, biopsy specimens were collected from the antrum of the stomach, and the *H. pylori* status was determined using the rapid urease test (Pronto Dry).

2.3. Gallbladder Processing. For histopathologic examination specimens were fixed in 10% buffered formalin and stained with a modified Giemsa stain. Under light microscopy curved, bent, pole-like, spiral, and fusiform bacteria were accepted as *H. pylori*-like bacteria (Figure 1). Immunohistochemical studies were also carried out on the fixed specimens (Figure 2). Tissue sections were placed on poly-L-lysine coated slides, and *H. pylori* antigen was determined according to the manufacturer's instructions (GeneTex, GTX 74404, San Antonio, TX, USA). Immunoreactivity was recorded as positive or negative.

Table 1: Rapid urease test results in gastric mucosa and gallbladder mucosa.

Rapid urease test	*H. pylori* positivity *N* (%)	*H. pylori* negativity *N* (%)	*P* value
Gastric mucosa	47 (58.7%)	33 (41.3%)	$P = 0.0001$
Gallbladder mucosa	21 (22%)	71 (78%)	
Both	15 (19%)	NA	NA

2.4. Statistical Analysis. Relationships between variables were tested by the Pearson and Spearman correlation coefficients (Giemsa, IHC, and rapid urease test), and McNemar's test was used to compare the rapid urease test in the gallbladder mucosa and the rapid urease test in the gastric mucosa; $P < 0.05$ was considered as statistically significant.

3. Results

Ninety-four patients with symptomatic gallstones undergoing laparoscopic cholecystectomy were enrolled for the study. Eighty of these patients had agreed to undergo gastroscopy before surgery. The rapid urease test in the gastric mucosa was negative in 33 (41.3%) and positive in 47 (58.7%) of these patients. The rapid urease test was positive in the gallbladder mucosa of 21 patients (22%). In 15 patients both gastric and gallbladder mucosa tested positive with the urease test. In 26 patients the gastric urease test was positive while the gallbladder mucosa tested negative. In only two patients the gallbladder mucosa tested positive while the gastric mucosa was negative. In four patients with a positive urease test in the gallbladder mucosa a gastric endoscopy was not done due to the patient's refusal. There was a significant correlation between the rapid urease test in the gallbladder mucosa and in the gastric mucosa ($P = 0.0001$, Table 1).

Laparoscopic cholecystectomy was completed uneventfully in all 94 patients with no serious postoperative complications. On histopathologic examination after Giemsa staining, *H. pylori*-like bacteria were detected in 25 patients

Table 2: Detection of *H. pylori* presence with different tests.

Tests	*H. pylori* positivity *N* (%)	*H. pylori* negativity *N* (%)	*P* value
Rapid urease test	21 (22%)	73 (78%)	
IHC	17 (18%)	77 (82%)	$P = 0.0001$ $r_s = 0.590$
Giemsa staining	25 (27%)	69 (73%)	
Triple positivity	12 (13%)	NA	NA

(27%), and with immunohistochemical study, *H. pylori* antibodies were detected in 17 (18%). In 12 patients all three tests were positive. Urease positivity alone was seen in 8 patients, while Giemsa staining was the only positive test in 10 patients. Immunohistochemical positivity as the only positive test was seen in only one patient. Overall in 35 patients (37%) gallbladder mucosa tested positive for *H. pylori* with any one of the three tests. Correlation of three tests Giemsa, IHC, and rapid urease test was significant (r_s: 0.590, $P < 0.001$, Table 2). Twenty-four patients had cholesterol stones (25.5%), 32 had pigment stones (34.5%), and 38 (40%) had mixed stones. There was no significant relationship between stone type and *H. pylori*. Cultures taken from the gallbladder mucosa grew microorganisms in 38 patients (40%). 13 different microorganisms were isolated, *E. coli* being the most common.

4. Discussion

Overall, in 35 of 94 patients undergoing cholecystectomy for symptomatic gallbladder stones, the gallbladder mucosa tested positive for *H. pylori* with any one of the three tests used in this study. This positivity was commonly associated with the presence of *H. pylori* in the gastric mucosa.

Detection of the presence of *H. pylori* in bile can be done with different methods, which are far from being perfect. The best way to show *H. pylori* is to grow *H. pylori* in cultures, but *H. pylori* are extremely hard to culture due to the microaerophilic properties of this microorganism, which die when they contact air. In another study it was shown that various PCR techniques could be used as a method for detection of *H. pylori* DNA in bile [16]. Fallone et al. [17] failed to find DNA of the genus *Helicobacter* in the bile of Canadian patients with biliary disorders, whereas a completely contrary result was described by Silva et al. (who found bacterial nucleotide sequences in most Brazilian subjects with similar diseases) [18]. Regional differences due to variable rates of infection and the changing sensitivity of the various PCR techniques used may be responsible for the difference in the reported studies [19].

The rapid urease test is easy to use and reliable. This test was positive in 58.7% of the gastroscopies performed in our study. In 15, the urease test was positive in both the gastric and gallbladder mucosa. On the other hand, of 47 patients with *H. pylori* in the stomach, the gallbladder showed the presence of *H. pylori* in 20 patients who tested positive with any one of the three tests used in this study (43%) revealing the close relationship of the common presence of *H. pylori* in both organs. Gallbladder mucosa in only two patients tested positive for the urease test while the stomach was negative for *H. pylori*. No signs of *H. pylori* were detected in the gallbladder in 59 of the 94 patients operated.

With the Giemsa stain, *H. pylori*-like bacteria were found in 27% of 94 gallbladder specimens while *H. pylori* antibodies were detected in 18% by immunohistochemistry. Chen et al., using W-S silver stain and light microscopy, showed *H. pylori*-like bacteria in 13.6% of cholecystectomy specimens, while only 7.1% tested positive by immunohistochemistry [13]. Detection of antibodies is important because this test is specific for *H. pylori*, and the other two tests used in this study also detect *H. pylori*-like organisms. With the Giemsa stain, *H. pylori*-like organisms were mainly seen on the surface of epithelial cells of the gallbladder mucosa and uncommonly in the intercellular zone or within the mucous gland. The *H. pylori* were seen as spiral, U- and S-shaped in morphology and were distributed in a scattered or aggregated fashion. All these characteristics are similar to those of *H. pylori* in the stomach.

Previous studies with different methods have revealed the presence of *H. pylori* in the biliary tract in 50–60% of patients studied [20–22]. *H. pylori* in the gastric mucosa is much more common. In Turkey and similar countries *H. pylori* is found in 80% of the population [23]. Our study and others clearly demonstrate that *H. pylori* can resist bile salts and can survive and colonize in the biliary tract. The route of infection, however, is not clear.

Monstein et al. have demonstrated *H. pylori* DNA in cholesterol gallstones and claimed the role of *H. pylori* in the etiology of cholesterol gallstones [8]. *H. pylori* infection of the gallbladder has been shown to increase the precipitation of cholesterol to form stones [24, 25]. Also it has been shown that urease induced calcium precipitation by *Helicobacter* species that may initiate gallstone formation [26]. We think that chronic *H. pylori* infection of the gallbladder may impair gallbladder contractility and so lead to increase in the precipitation of bile components to form stones. In our study no relationship was found for the presence of *H. pylori* in the gallbladder and the type of the gallstone.

This study demonstrates the presence of *H. pylori* in the gallbladder in 37% of patients with symptomatic gallstones. This study also demonstrates the concomitant presence of *H. pylori* in the gastric and gallbladder mucosa. However, it does not suggest *H. pylori* for the etiology of gallstones. Nevertheless, the effect of *H. pylori* eradication on the incidence of gallstones remains to be investigated.

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Research Article

Molecular Detection of Antibiotic Resistance in South African Isolates of *Helicobacter pylori*

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Rapid diagnosis and treatment of *Helicobacter pylori* (*H. pylori*) presents a challenge. We aimed at investigating the presence of *H. pylori*, susceptibility profile, and associated mutations in an effort to validate the effectiveness of GenoType HelicoDR assay in *H. pylori* typing in our environment. Two hundred and fifty-four biopsy specimens were cultured and DNA extracted from seventy-eight positive cultures using the Qiagen DNA extraction kit. The GenoType Helico DR which employs reverse hybridisation was used to confirm the presence of *H. pylori*, determination of its susceptibility to antimicrobials, and detection of mutations conferring resistance to clarithromycin and fluoroquinolones. The organism was isolated from 168/254 (66.1 %) of the specimens by culture. Of the 78 strains used for further investigation, 12/78 (15.38%) were resistant to clarithromycin while 66/78 (84.61%) were susceptible. For fluoroquinolone, 70/78 (89.74%) strains were susceptible while 8 (10.26%) were resistant. Mutations were observed in 17 strains with A2147G being the most prevalent; A2146C and D91N were the least. The reverse hybridisation assay is an easy and fast technique in confirming the presence of *H. pylori*, its antimicrobial profile, and associated mutations. Analysis regarding the suitability of this assay for *H. pylori* typing is warranted in other regions.

1. Introduction

The burden of *Helicobacter pylori (H. pylori)* presents a tremendous challenge therapeutically [1, 2]. Clinical management of *H. pylori* infection seems tenacious because the organism lives in an environment not easily accessible to many medications, the overwhelming presence of antibiotic resistance and poor patient compliance [3]. Eradication of the organism from the stomach results in significant remission from diseases related to the pathogen [2, 4]. Regimens of choice employed for eradication currently involve the use of combination therapies: a proton pump inhibitor (PPI) or bismuth compounds and two antibiotics most commonly clarithromycin and metronidazole and/or amoxicillin [5].

Clarithromycin currently remains the available most powerful antibiotic against *H. pylori* with very low minimum inhibitory concentration compared to other molecules [6]. Fluoroquinolone such as ciprofloxacin has been incorporated in the treatment regimens after repeated treatment failures and quinolone-based triple therapies have been shown to be highly effective to patients [3, 7]. However, *H. pylori* can become resistant to these compounds, which jeopardize the success of treatments [3, 8]. Particularly, resistance to clarithromycin in *H. pylori* isolates is regarded as a main cause of treatment failure in developing countries [6]. The organism is known for its wide genetic diversity which varies geographically [9], and hence antimicrobial susceptibility profiles also vary demographically. Resistance is high in naive patients and even higher in patients suffering from unsuccessful eradication therapy [8]. Resistance to clarithromycin has been linked to decrease binding of the macrolides to the 50S bacterial ribosomal subunit [6, 10]. Extensive studies have demonstrated that point mutations in the peptidyltransferase region encoded in domain V of 23S rRNA are responsible for the organisms' resistance to clarithromycin [4]. These mutations are able to inhibit the binding between clarithromycin

and the ribosomal subunit dedicated to the specific antibiotic related protein synthesis. Mutations frequently associated with clarithromycin resistance are the transitions in A2143C and A2142C positions of rRNA whilst substitution in A2142C is less frequent [6, 11, 12]. Different mutation types have been described from studies in different parts of the world amongst which are A2115G, G2141A, T2117C, T2182C, T2289C, G22-4A, C2245T, and C2611A [6]. Besides the low frequency, the clinical relevance of A2115G, G2141A, T2117C and T2289C is not well established [6, 7]. Quinolones exert their antimicrobial effects by affecting the A subunit of the DNA gyrase, the only known target enzyme in *H. pylori* [7, 13]. Resistance is associated with mutation in the gyr87 and gyr91 locus or complete absence of the wild type loci. Possible mutations found on loci 91 are D91N, D91G, D91Y, and D91A as well as N87H, N87I, N87K, or N87Y found on position 87 [3, 13, 14].

Resistance of *H. pylori* to antibiotics is currently widely determined in clinical bacteriology laboratories by standard methods, such as disk diffusion, microbroth dilution assay or Etest [15, 16]. These phenotypic methods are efficient in discriminating between susceptible and resistant strains, but results can be obtained only after several days (considering that *H. pylori* needs about 3–7 days to grow) and do not give insight to the type of mutation present, which could be of epidemiological and clinical significance. Conventionally, mutations are detected using molecular typing schemes such as PCR restriction fragment length polymorphism (PCR-RFLP) and sequencing [4, 11, 12, 17]. In the literature, however, there is a dearth of knowledge on mutations that occur at codons 2146-2147 for clarithromycin resistant strains [18]. A PCR-based hybridization method (Hain Life Sciences, Nehren, Germany) using a strip designed for detection of *H. pylori* and mutations at codons 2146-2147 (A2146G, A2146C, and A2147G) in 23S rRNA gene in clarithromycin resistant isolates and mutations at codon 87 and 91 (N87K, D91N, D91G and D91Y) which are associated with resistance to fluoroquinolone were employed and validated in a study by Cambau et al. [18] in France. This reverse hybridisation assay offers a one-step detection of the presence of *H. pylori,* its antimicrobial profile, and mutations associated to clarithromycin and fluoroquinolone. We sought therefore to employ the use of this assay to investigate the presence of *H. pylori* and associated mutations to these antibiotics in an effort to validate its effectiveness in *H. pylori* related studies in the environment of the Eastern Cape province of South Africa due to its high prevalence reported in our previous studies [2, 16].

2. Materials and Method

2.1. Bacterial Strains and Minimum Inhibitory Concentration. In this study, 168/254 (66.1%) of the specimens collected from patients who underwent endoscopic examination for upper gastrointestinal problems with no history of treatment with macrolide and fluoroquinolone antibiotics were found positive for culture. Seventy-eight of the positive strains isolated from 50 males and 28 females were used for further investigations. Antral and corpus gastric mucosal biopsy specimens were taken from each dyspeptic patient. The biopsies were immediately placed in sterile bijou bottles containing 0.2 g/L of cysteine and 20% glycerol in brain heart infusion (BHI) broth and transported in ice to the laboratory within 2 h of collection for culture [2].

Biopsies were homogenised under aseptic conditions in 0.2 g/L of cysteine and 20% glycerol in BHI broth and a loop full plated primarily on freshly prepared Columbia agar base (Oxoid, Basingstoke, England) supplemented with 7% sheep's blood (Oxoid, England) and Skirrow's supplement (Oxoid, England); trimethoprim (2.5 mg), vancomycin (5 mg), cefsulodin (2.5 mg), and amphotericin (2.5 mg) were also added to the medium. All plates were incubated at 37°C for 3–5 days under microaerophilic conditions (5%-6% O_2 , 10% CO_2 , 80%–85% N_2) (Anaerocult, Basingstoke, England). Isolates were identified based on colony morphology and positive oxidase, urease, catalase tests, and confirmation by amplification of the *glmM* gene as previously reported [2]. Confirmed isolates were suspended in 20% glycerol and stored at −80°C in a freezer (Sanyo, Japan) until genotyping was performed. *H. pylori* reference strain NCTC 11638 was included in all experiments. Approval for this study was obtained from the Research Ethics Committee of the University of Fort Hare and the Eastern Cape Department of Health (protocol number EcDoH-Res 0002).

Minimum inhibitory concentration (MIC) was determined for clarithromycin and ciprofloxacin as previously described [16]. MIC values for the antibiotics were 0.0625–256 μg/mL for ciprofloxacin and 0.125–256 μg/mL for clarithromycin.

2.2. PCR Method. DNA was extracted from 78 strains using QIAamp tissue kit (Qiagen DNA extraction kit, SA) following the manufacturer's recommendation closely. Amplification of the bacterial DNA was done using hot-start DNA polymerase (Hain Lifescience, Nehren, Germany). Biotinylated primers were used for this study and were provided in the amplification kit. Primers were designed using the gene sequence from GenBank accession number NC_009151. Polymerase chain reaction for a single mixture had a final volume of 50 μL containing 35 μL primer/nucleotide mix (PNM), 5 μL 10x polymerase incubation buffer, 2 μL of 1.5 mM $MgCl_2$, 3 μL of nuclease free water (Hain Lifescience, Nehren, Germany) 0.2 μL Thermo-Start Taq DNA polymerase (1-2 units were added to each tube), and 5 μL DNA template. PCR was performed with a thermal cycler (Applied Biosystem, SA). The amplification cycles consisted of an initial hot start of 95°C for 15 min, initial denaturation of target DNA at 95°C for 5 min, denaturation at 95°C for 30 sec and 58°C for 2 min, primer annealing at 95°C for 25 sec, 53°C for 40 sec, and 70°C for 40 sec, and extension at 70°C for 8 min. All reactions were performed through 32 cycles (Hain Lifescience, Nehren, Germany).

2.3. GenoType HelicoDR Analysis. Confirmation of isolates as *H. pylori,* antimicrobial susceptibility, and mutational analysis to clarithromycin and fluoroquinolone was performed using the GenoType HelicoDR kit (Hain Lifescience,

Nehren, Germany). The kit employs the use of reverse hybridisation performed using hybridisation trays and TwinCubator (Hain Lifescience, Nehren, Germany) according to the manufacturer's instructions. Briefly, 20 μL of amplified DNA was denatured and added to biotinylated probes on the strip and the hybrids formed were detected by enzyme-linked immunosorbent assay (ELISA) upon addition of substrate conjugate and substrate. Four gyr87 wild type probes (gyr87WT1–gyr87WT4) and one mutant probe (gyr87MUT), one wild type probe (gyr91WT1), and three mutant probes (gyr91MUT1–gyr91MUT3) were used for detecting fluoroquinolone resistance at position 87 and 91, respectively. For clarithromycin, one wild type probe (23SWT) and three mutant probes (23SMUT1–23SMUT3) were used for detecting resistance. On the strip, were designated conjugate control (CC), amplification control (AC) and *H. pylori* (HP). When one of the WT probes stained positive together with the gyr91WT as well as 23SWT and no mutation band formed, the results were interpreted as susceptible to the respective antibiotic. The presence of a band at CC and AC meant that the conjugate control and amplification control were in the right frame while at HP implied presence of *H. pylori* according to the manufacturer's instruction (Hain Lifescience, Nehren, Germany).

2.4. Statistical Analysis. Epi Info version 2000 (Center for Disease Control and Prevention, Atlanta, GA., USA) was used for statistical analysis. Chi square or Fischer exact test was applied to test whether differences in susceptibility/resistance between values of males and females were significant at P value <0.05. The sensitivity and specificity of the GenoType HelicoDR kit for detection of resistance to clarithromycin and fluoroquinolone, respectively, were calculated as previously described [18].

3. Results

3.1. Helicobacter pylori Strains. In this study, 168/254 (66.1%) specimens were positive for *H. pylori* by culture and confirmed using polymerase chain reaction/reverse hybridisation assay (GenoType HelicoDR). Seventy-eight of the 168 strains were used for further investigations.

3.2. Antimicrobial Susceptibility and Mutational Analysis. Seventy (89.7%) of the 78 strains were susceptible to fluoroquinolone while eight (10.3%) were resistant. For clarithromycin, 66/78 (84.6%) were susceptible and 12/78 (15.4%) were resistant. The sensitivity and specificity of detecting resistance were 98% and 100% for clarithromycin and 89% and 93% for fluoroquinolone, respectively.

Of the 78 strains employed for further analysis, 28 (35.9%) were from females and 50 (64.1%) from males. Prevalence of clarithromycin resistance in females and males was 32.1% (9/28) and 6% (3/50), respectively, while for fluoroquinolone it was 17.9% and 6%, respectively. A higher prevalence of resistant isolates was observed in female compared with male subjects in this investigation. There was statistically significant difference with the use of clarithromycin for both sexes ($P = 0.006$), although not statistically significant for fluoroquinolone ($P = 0.127$).

Some mutations designed in line with the kit to detect resistance to clarithromycin and fluoroquinolone were delineated. Mutations observed in 17 strains are summarised in Table 1.

Three strains had 2 or more mutations with the highest number of mutations (4) occurring in 252C. A2147G mutation associated with resistance to clarithromycin was the most prevalent mutation type in this study while A2146C and D91N were the least. Twelve of the 17 strains studied possessed A2147G mutation (Table 2).

The frequency of A2146C mutation was very low occurring in only one (252C) of 17 strains (5.8%). The MIC of clarithromycin for these mutants (A2147G and A2146C) ranged from 32 to 256 μg/mL.

Of the 8 strains found to be resistant to fluoroquinolone, all (100%) possessed N87K mutation associated with fluoroquinolone. No designated mutation was found in five strains (247C, 253C, 369A, 249A, and 249C) using this assay as there was the complete absence of the gyrase 87 wild type indicating a mutation (designated as N87K mutation). Also, D91N associated with resistance to fluoroquinolone was detected in 1 (5.8%) of the 17 strains whilst D91Y and other designated mutation associated with fluoroquinolone resistance were not found. Minimum inhibitory concentration for fluoroquinolone (247C, 253C, 369A, 249A, and 249C) (Table 1), ranged from 8 μg/mL to 32 μg/mL. Strains with mutations for both clarithromycin and fluoroquinolone (252A and 252C) had MIC 32 μg/mL while 247A had 256 μg/mL.

4. Discussion

Clarithromycin and fluoroquinolone are presently the drugs of choice employed for triple combination therapy in the treatment of *H. pylori* infection [6]. Resistance to these drugs is emerging and presents a challenge. Different studies all over the world have reported resistance to clarithromycin and fluoroquinolone [8, 12, 13, 19]. The high resistance rates to these antibiotics, the burden of *H. pylori* infection, and its associated disease conditions coupled with the difficulties of rapid diagnosis and management of patients [16] necessitated the determination of the antibiogram and associated mutation to clarithromycin and fluoroquinolone to *H. pylori* strains isolated from the Eastern Cape province known to have a high prevalence of *H. pylori*-related morbidities [2, 16] using the GenoType HelicoDR assay.

H. pylori was detected in 168/254 (66.1%) of the specimens studied. Clarithromycin is used worldwide as one of the potent antibiotics in the eradication therapy of *H. pylori* [4, 17]. However, resistance to clarithromycin has been increasingly reported in several studies [12, 17]. This led to the introduction of new treatments such as the fluoroquinolones which seemingly is offering great hope, but unfortunately resistance to them is emerging [7, 13]. The presence of resistance is often associated with failure of eradication therapy [20]. Findings from this study revealed moderate rate of resistance to clarithromycin and fluoroquinolone with

Table 1: Mutations associated with resistance to clarithromycin and fluoroquinolone.

Mutant strain	Number of independent mutations analysed	Mutant alleles[a]		
		Designation	Amino acid change	MIC (μg/mL)
245A	1	*23SMUT3*	A2147G	256
245C	1	*23SMUT3*	A2147G	32
247A	2	*23SMUT3* No *gyr 87* WT	A2147G N87K	256
247C	1	No *gyr 87* WT	N87K	8
249A	1	No *gyr 87* WT	N87K	32
249C	1	No *gyr 87* WT	N87K	16
252A	2	No *gyr 87* WT *23SMUT3*	N87K A2147G	32
252C	4	No *gyr 87* WT *gyr 91* WT *23SMUT2* *23SMUT3*	N87K D91N A2146C A2147G	32
253C	1	No *gyr 87* WT	N87K	16
254A	1	*23SMUT3*	A2147G	256
119A	1	*23SMUT3*	A2147G	32
275A	1	*23SMUT3*	A2147G	128
305A	1	*23SMUT3*	A2147G	64
305C	1	*23SMUT3*	A2147G	128
369A	1	No *gyr 87* WT	N87K	16
436C	1	*23SMUT3*	A2147G	256
499C	1	*23SMUT3*	A2147G	64

[a]Distinct mutation identified is given an allele name (designation), which represents a specific change at the nucleotide (nt) in a gene and a corresponding amino acid change in the gene product.

Table 2: Distribution of clarithromycin and fluoroquinolone mutations amongst 17 strains.

Mutations	Total number of strains	% of strains with each mutation type
A2147G	12	70.5 (12/17)
A2146C	1	5.8 (1/17)
N87K	8	47.05 (8/17)
D91N	1	5.8 (1/17)
Total number of mutation types	22	

percentages of 15.38% and 10.26%, respectively. This result corroborates the finding of Kim et al. [12] who reported resistance to clarithromycin with a range of 7.6% to 18.6% in Korea. Macrolides like clarithromycin are expensive; however, cross-resistance linked with the use of other less expensive macrolides may be responsible for this resistance. Worthy of note is the fact that clarithromycin susceptible and resistant strains have been isolated from patients with no history of exposure to macrolides [21]. Therefore, it is imperative to guide empiric treatment since administration of clarithromycin can be selected for resistance.

The moderate rate of resistance of 10.26% to fluoroquinolone reported in this study ties with the findings of Wang et al. [13] who reported 15.6% resistance to ciprofloxacin in their study in Alberta, Canada. However, generally low resistance to the fluoroquinolones has been reported compared to other antibiotics. For example, in our previous study [16], all strains were susceptible to ciprofloxacin as opposed to the 10.26% resistance generally reported for the fluoroquinolone in the current study. We may relate this to the difference in strains as well as the methods (phenotypic versus molecular) used in both studies. Also, the strips in the GenoType HelicoDR assay are designed to generally target the fluoroquinolones. Hung et al. [3] also documented 5.7% resistance of their strains to ciprofloxacin, and Kohanteb et al. [22] reported 4.7% in their study. Isolates from Belgium, France, Italy, and Germany have higher resistance rates to ciprofloxacin or levofloxacin ranging between 16.8% and 23% [3, 23]. Also, higher resistance rates (ciprofloxacin: 33.8%; levofloxacin: 21.5%) have been observed in Japan [19]. These disparate rates of resistance could be attributed to geographical region and drug usage differences [9].

The prevalence of antibiotic resistance was higher in males than in females in this study reaching statistical significant difference for clarithromycin ($P = 0.006$), but not for fluoroquinolone. Importantly, more males were recruited for this study than females and this could account for the difference observed. Noteworthy is the fact that the number of strains resistant to clarithromycin (12) and fluoroquinolone

(8) is almost the same, suggesting that these antibiotics could be close in their suitability as drugs of choice for *H. pylori* treatment in our environment corroborating our previous finding [16].

Mutations associated with resistance to these antibiotics were investigated. Three strains showed 2 or more mutations with the highest number of mutations occurring in 252C (Table 1). We observed that the higher the number of mutations per strain, the higher the MIC value of that strain. Strains in our study which were resistant to clarithromycin with mutation A2147G had MIC values that ranged from 32 to 256 μg/mL. Strains 252A and 252C had MIC of 32 μg/mL, respectively, while 247A had MIC 256 μg/mL; they all possessed multiple mutations; 247C and 253C had MIC values of 8 μg/mL and 16 μg/mL (Table 1), respectively. These variations could be due to strain diversity.

Fluoroquinolone acts by inhibiting DNA gyrase, topoisomerase, and interfering with bacterial DNA replication; since topoisomerase is not found in the *H. pylori* genome, mutation in the *gyrA* gene which encodes DNA gyrase is considered to be the major cause of resistance to fluoroquinolones [7]. The codons N87 and D91 are recognised as the most important target sites for ciprofloxacin binding [3, 13]. The N87H, N87I, N87K, and N87Y as well as D91G, D91N, and D91Y mutations in *gyrA* have been reported in fluoroquinolone-resistant *H. pylori* strains [3]. The assay used in this study was designed to depict N87K, D91N, D91G, and D91Y which have been frequently reported. However, N87K and D91N mutations were the only mutations associated with fluoroquinolone found in this study. N87K was the most prevalent mutation (8/17; 47.05%) associated with fluoroquinolone amongst our strains whilst D91N was found in one strain (Table 2).

Our findings are in line with those of Wang et al. [13] and Hung et al. [3] who reported the presence of these mutations in their studies, respectively. Furthermore, it confirms the fact that these mutations are the most frequently found mutations [3, 24]. N87K was found in eight strains. The D91Y and D91G mutations were not found in the current study. This contradicts the finding of Garcia et al. [14] who reported the presence of these mutations in six and seven strains, respectively. However, occurrence in just five and two strains of their huge sample size may imply a generally low occurrence.

Clarithromycin acts by inhibiting protein synthesis by binding to the peptidyltransferase loop of 23S rRNA which has been shown at residues A2058 and A2059 in the 23S rRNA gene of *E. coli*. When mutation occurs in these residues, the binding affinity of clarithromycin to ribosomes is reduced, resulting in clarithromycin resistance [11, 21]. The assay used in this study was designed to target the presence of A2147G, A2146G, and A2146C associated with clarithromycin resistant strains [18]; there is a dearth of information in the literature on A2147G, A2146G and A2146C mutation compared to A2142G, and A2143G which are frequently reported [6, 7] to be associated with clarithromycin resistance [12].

Twelve (70.5%) of the 17 strains reported to be resistant in this study had A2147G mutation (Tables 1 and 2). This accords with the findings of Cambau et al. [18] who reported a high prevalence of A2147G mutation amongst their strains. The high occurrence of A2147G mutation suggests its high frequency amongst our local strains as opposed to A2146C which occurred in one (252C) of the 17 strains studied. The sensitivity and specificity of detecting resistance to clarithromycin and fluoroquinolone observed in our current investigation corroborate with those of Cambau et al. [18], although they specifically used levofloxacin, which is also a member of the fluoroquinolone. They reported values of 94% and 99% for clarithromycin and 87% and 99% for levofloxacin, respectively.

In conclusion, this study revealed a moderate rate of resistance to fluoroquinolone and clarithromycin, with A2147G and N87K being the main mutations associated with clarithromycin and fluoroquinolones, respectively. However, continuous surveillance of fluoroquinolone and clarithromycin resistance in *H. pylori* is thus relevant in this area to guide empiric treatment.

Ethical Approval

This study was approved by the Eastern Cape Department of Health (Protocol no. EcDoH-Res 0002) and the Research Ethics Committee of the University of Fort Hare, Alice, South Africa.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Review Article

Is *Helicobacter pylori* Infection the Primary Cause of Duodenal Ulceration or a Secondary Factor? A Review of the Evidence

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Helicobacter pylori (*H. pylori*) has a role in the multifactorial etiology of peptic ulcer disease. A link between *H. pylori* infection and duodenal ulcer disease is now established. Other contributing factors and their interaction with the organism may initiate the ulcerative process. The fact that eradication of *H. pylori* infection leads to a long-term cure in the majority of duodenal ulcer patients and the fact that the prevalence of infection is higher in ulcer patients than in the normal population are cogent arguments in favor of it being the primary cause of the ulceration. Against this concept there are issues that need explanation such as the reason why only a minority of infected persons develop duodenal ulceration when infection with *H. pylori* is widespread. There is evidence that *H. pylori* infection has been prevalent for several centuries, yet duodenal ulceration became common at the beginning of the twentieth century. The prevalence of duodenal ulceration is not higher in countries with a high prevalence of *H. pylori* infection. This paper debate puts forth the point of view of two groups of workers in this field whether *H. pylori* infection is the primary cause of duodenal ulcer disease or a secondary factor.

1. It Is the Primary Cause of Ulceration (N. Ananthakrishnan and Vikram Kate)

1.1. Helicobacter pylori Is the Primary Cause of Duodenal Ulcer. The isolation of *Helicobacter pylori (H. pylori)* from the gastric mucosa generated excitement when it was postulated by Marshall that these microorganisms could be the cause of gastritis and play an important role in the etiology of peptic ulcer disease [1]. *H. pylori* infection is almost always associated with an inflammatory response; however, peptic ulcer disease and gastric carcinoma occur only in a subset of individuals chronically infected with *H. pylori*. Presumably, both bacterial and host factors contribute to this differential response.

The role of *H. pylori* as a gastric pathogen is dependent on virulence factors and pathogenic mechanisms. Virulence factors are those that allow *H. pylori* to survive in the hostile environment of the gastric lumen which includes its spiral shape, motility, adaptive enzymes, proteins, and ability to adhere to gastric mucosal cells and mucus [2]. Pathogenic mechanisms are those that lead either directly to disruption of the gastric mucosal barrier including its toxins like Vac A and Cag A and mediators of inflammation.

1.2. Pathogenic Mechanisms and Virulence Factors. The spiral shape and flagella of the organism allow efficient motility in the mucus and in the gastric juice. The enzyme urease by breaking down urea in the gastric juice appears to generate enough bicarbonate and ammonium ions around the organism to allow its safe passage through the gastric acid barrier to reach the protective mucous layer. Ammonia elevates the pH of the gastric mucous layer from about 6 to 7 [3]. It is known to deplete aerobic cells of alpha keto-glutarate, an essential substrate for the tricarboxylic acid cycle. Ammonia in high concentration induces vacuoles exactly the same as those seen when cells are exposed to the Vac A toxin of *H. pylori* [4]. Once within the gastric mucus, *H. pylori* is

able to attach itself to phospholipids such as phosphatidyl ethanolamine, sialylated glycoproteins such as ganglioside monosialic 3 (GM3), and Lewis B antigens present in persons with blood group O [5, 6]. Once attached to the mucus layer and the mucosa, *H. pylori* secretes soluble proteases and phospholipase, which may be harmful to both the integrity of the mucus layer and the underlying cells. The "wettability" of gastric mucus is increased when *H. pylori* is present perhaps due to partial lysis of the phospholipid component [7].

One of the most important aspects of *H. pylori* pathogenicity is the "vacuolating cytotoxin" which is expressed in nearly all patients with *H. pylori* associated duodenal ulcer [8, 9]. The marker for cytotoxin is a gene for the cytotoxin protein called Vac A. A second protein at 127 kDa is called cytotoxin-associated gene A or Cag A. Cag A is a marker for the vacuolating toxin effect and the gene for Cag A is only present when VacA cytotoxin effect is present. The organisms have been classified into type I organisms which have Cag A and Vac A which are more ulcerogenic and type II organisms that lack Cag A and do not produce cytotoxins [8, 9]. Antibodies to the toxin are present in nearly all duodenal ulcer patients. This is one of the factors which determine that all patients with *H. pylori* do not have duodenal ulcer disease.

Recently, a novel virulence factor, duodenal ulcer promoting gene A (dupA), has been identified and found to be associated with disease in some populations. A recent meta-analysis investigated the relationship of dupA genotypes and *H. pylori*-related clinical outcomes by using previous reports of 2,358 patients from around the world with dupA-positive genotypes. It was found that in 48%, dupA was associated with duodenal ulcer ($P = 0.001$, odds ratio OR = 1.4, confidence interval CI = 1.1–1.7); however, the prevalence of dupA positivity and its association with disease differed among the various regions around the world [10]. In another study from India, a total of 140 *H. pylori* strains isolated from duodenal ulcer ($n = 83$) and nonulcer dyspepsia (NUD) patients ($n = 57$) were screened by PCR and dot-blot hybridization to determine the presence of the open reading frames (ORFs) "*jhp0917* and *jhp0918*" [11]. The PCR and dot-blot results indicated the presence of "*jhp0917* and *jhp0918*" in 37.3% (31/83) and 12.2% (7/57) of *H. pylori* strains isolated from duodenal ulcer and nonulcer dyspepsia patients, respectively. The prevalence of dupA was significantly greater among strains isolated from patients with duodenal ulcer than from patients with NUD in this population ($P = 0.001$, odds ratio = 4.26, confidence interval = 1.60–11.74). The authors suggested that dupA can be considered a biomarker for duodenal ulcer patients in India.

Levi et al. reported increased gastrin levels due to *H. pylori* infection which induced increased gastric acid secretion leading to duodenal ulcer [12]. Eradication of *H. pylori* abolishes the hypergastrinemia suggesting that this is due to *H. pylori* infection.

Somatostatin deficiency is seen in the gastric antrum in patients infected with *H. pylori* [13]. Subsequently, it was discovered that immunoreactive somatostatin, D cells, and somatostatin message were all decreased in patients with gastritis [13, 14]. As a result of either genetic predisposition or an alteration in G-cell or D-cell function due to *H. pylori* infection, some patients will develop an increased parietal mass. The increased parietal mass results in an increased acid load that leads, in some patients, to gastric metaplasia in the duodenum. *H. pylori*-associated antral gastritis appears to be prerequisite for colonization of areas of duodenal metaplasia and the appearance of duodenitis and duodenal ulceration [14]. In patients with *H. pylori*- related duodenal ulcer it was found that duodenal colonization by *H. pylori* in patients with nonulcer dyspepsia is strongly predictive of the subsequent development of duodenal ulcer [15]. These factors provide a link between *H. pylori*, gastritis, acid hypersecretion, and peptic ulceration.

1.3. Role of H. pylori in Duodenal Ulcer Disease

1.3.1. Uncomplicated Duodenal Ulcer. Studies have shown that *H. pylori* has a role in the multifactorial etiology of peptic ulcer disease and there is interplay of many factors such as the acid attack and the mucosal defence [12, 14, 16]. It is modulated by genetics, gender, blood group, smoking, age, and various physiologic considerations, including acid output [16]. These and other considerations explain the discrepancy between the high frequency of *H. pylori* infection in the population and a less than 10% overall lifetime prevalence of duodenal ulcer disease.

The prevalence of *H. pylori* infection in duodenal ulcer has consistently been found to be between 90% and 100% [17, 18]. In our earlier report we found that patients with duodenal ulcer had a significantly higher prevalence of *H. pylori* at 91% compared to normal controls [19]. Most agents used for treatment of duodenal ulcer are aimed at reducing acid secretion and promote healing by minimizing acid attack; however, this antisecretory therapy has no effect on the *H. pylori* status and does not correct the underlying state of gastroduodenitis. The mucosa, therefore, remains abnormal and vulnerable to ulcer relapse following cessation of antisecretory therapy. Therefore, treatment that fails to address the role of *H. pylori* in the causation of the mucosal inflammation, which predisposes to ulceration, is likely to confer only short-term benefit.

Eradication of infection has been shown beyond doubt to markedly alter the natural history of duodenal ulcer disease. A number of series have shown either low or no recurrence of ulcer at the end of one year compared with a natural recurrence rate of more than 70% [20, 21]. Several studies, the Maastricht III Consensus Report and the Second Asia-Pacific Consensus Guidelines for *Helicobacter pylori* infection, have demonstrated that ulcers recur in only a small percentage of patients following successful *H. pylori* eradication in comparison to a recurrence rate of 50% or greater within the course of one year when the organism persists and hence *H. pylori* eradication is recommended in patients with duodenal ulcer disease [22–28]. In a Cochrane Systematic Review, it was concluded that *H. pylori* eradication therapy is effective treatment for *H. pylori* positive peptic ulcer disease [29]. However, eradication has proved to be difficult and reinfection rates are high in developing countries. Recrudescence or reinfection with *H. pylori* is common and may be an important factor in recurrence of peptic ulcer disease

since successful eradication virtually abolishes recurrence of duodenal ulcer [30]. Improper selection of therapy such as nitroimidazole comprising regimens in developing countries or factors such as low compliance due to use of complex regimen lead to a low efficacy of the therapy resulting in recrudescence or reinfection. Recrudescence is more often the cause of recurrence of peptic ulcer rather than recurrence following successful eradication.

The previous reports corroborate the view that *H. pylori* infection is the most important determinant in preventing ulcer relapse in patients with duodenal ulcer and supports its role as a primary factor in duodenal ulcer disease.

1.3.2. Helicobacter pylori and Complicated Duodenal Ulcer Disease. The major complications of duodenal ulcer disease are bleeding, perforation, and gastric outlet obstruction.

Bleeding. Bleeding is by far the most frequent complication of chronic duodenal ulcer disease. 30% of bleeding ulcers bleed massively [31]. Most of the studies report a high prevalence of *H. pylori* in bleeding duodenal ulcer. Kadayifei reported a higher prevalence rate of *H. pylori* infection in patients with bleeding duodenal ulcer (88%) compared to patients with uncomplicated duodenal ulcer (67.2%) [32]. He recommended eradication therapy for *H. pylori* for all patients with *H. pylori* positive duodenal ulcer to prevent recurrent bleeding. We too found a significantly higher prevalence of *H. pylori* in patients with bleeding duodenal ulcer at 89% when compared to controls at 60% [33]. Studies have shown that persistent *H. pylori* infection was an independent predictor of recurrence of duodenal ulcer bleeding [34–36]. It has also been seen that Cag A positive *H. pylori* infection is associated with an increased risk of bleeding [37].

In another Spanish study of 103 patients with bleeding duodenal ulcer, it was found that at a median followup of 27 months there were no instances of rebleeding in any of the 93 eradicated patients suggesting that bleeding from duodenal ulcer is virtually abolished if patients receive *H. pylori* eradication therapy [38]. A recent study reported that peptic ulcer rebleeding virtually does not occur in patients with bleeding duodenal ulcers following *H. pylori* eradication [39].

From the present data available in the literature it appears that persistence of *H. pylori* infection is one of the most important factors causing rebleeding in patients with bleeding duodenal ulcer and hence eradication therapy should be recommended as a routine in all patients with bleeding duodenal ulcer positive for *H. pylori* infection.

Gastric Outlet Obstruction. Although the conventional surgery of vagotomy and drainage is commonly performed for this complication of duodenal ulcer, some authors have advocated endoscopic balloon dilatation and *H. pylori* eradication for this group [40, 41]. Lam et al. recommended endoscopic dilatation with *H. pylori* eradication for patients with duodenal ulcer and gastric outlet obstruction [42]. We had found a high prevalence of *H. pylori* in patients with duodenal ulcer and gastric outlet obstruction [43]. This was true whether or not the ulcer was active or cicatrized. Gisbert and Pajares recommended in a review article on *H. pylori* and gastric outlet obstruction that treatment should start pharmacologically with the eradication of *H. pylori* whereas dilatation or surgery should be reserved for patients who do not respond to medical therapy [44]. These reports suggest that although a dilatation is carried out to relieve benign gastric outlet obstruction, eradication of *H. pylori* is an important component of this therapy as obstructed ulcers are also associated with *H. pylori*.

Perforation. In the last few years reports are being published on the role of *H. pylori* in perforated duodenal ulcer [45–49]. Earlier reports on the association between *H. pylori* and perforated duodenal ulcer in the nineties suggested that the prevalence of *H. pylori* was high in patients with perforated duodenal ulcer [50–52].

With the association of *H. pylori* infection with perforated duodenal ulcer postulated by many studies, attention was focused on the effect of eradication of the organism on the ulcer recurrence following simple closure of perforated duodenal ulcer [45–49, 53, 54]. In an earlier study from our institute on a prospective group of 202 patients and a retrospective group of 60 patients who had undergone simple closure of perforated duodenal ulcer it was found that at every interval of followup *H. pylori* infection rate was significantly higher in patients who had recurrent or residual ulcer [55]. Metzger et al. suggested that an immediate and appropriate *H. pylori* eradication therapy for perforated duodenal ulcers reduces the relapse rates after simple closure [56]. In a recent study on 150 patients with perforated duodenal ulcer following simple closure included on a prospective basis, we found that presence of recurrent ulcer was 18.6% in the eradicated patients when compared to 70% in noneradicated patients suggesting that *H. pylori* eradication reduces risk of ulcer recurrence after simple closure [57].

In a recent systematic review and meta-analysis on *H. pylori* eradication therapy after simple closure of perforated duodenal ulcer, the pooled incidence of 1-year ulcer recurrence in the *H. pylori* eradication group was 5.2% (95% confidence interval (CI) of 0.7 and 9.7), when compared with that of the control group (35.2%) with 95% CI of 0.25 to 0.45 [47]. The pooled relative risk was 0.15 with 95% CI of 0.06 to 0.37. The authors concluded that *H. pylori* eradication after simple closure of duodenal ulcer perforation gives better results than antisecretory noneradication therapy for prevention of ulcer recurrence and hence should be recommended for all infected patients.

From all the previously mentioned data it appears that *H. pylori* infection does play an important role in perforated duodenal ulcer and eradication is recommended in all infected patients following simple closure to prevent ulcer relapse.

1.4. Conclusion. The strong evidence in the literature linking *H. pylori* etiologically to duodenal ulcer and reports on eradication therapy of *H. pylori* in preventing relapse of uncomplicated and complicated duodenal ulcer suggest that *H. pylori is the primary cause of duodenal ulcer.*

2. It Is Not the Primary Cause but a Secondary Factor Delaying Healing (Frank I. Tovey)

The fact that eradication of *H. pylori* infection leads to a long-term cure in the majority of duodenal ulcer patients and the fact that the prevalence of infection is higher in ulcer patients than in the normal population are cogent arguments in favor of it being the primary cause of the ulceration. Against this concept there are difficulties that need explanation.

(a) Infection with *H. pylori* is widespread yet only a minority of infected persons develop duodenal ulceration [58].

(b) There is evidence that *H. pylori* infection has been prevalent for several centuries; yet duodenal ulceration became common at the beginning of the twentieth century [59–66].

(c) The prevalence of duodenal ulceration is not higher in countries with a high prevalence of *H. pylori* infection as would be expected if it were causal. Geographically the prevalence of duodenal ulceration does not correspond to the prevalence of *H. pylori*. Within countries with the same overall prevalence of *H. pylori* infection the prevalence of duodenal ulceration may vary from region to region [67–81].

One explanation of these problems is that virulent strains of *H. pylori* have emerged and could account for the onset of duodenal ulceration at the turn of the twentieth century and for the geographical variations in the prevalence of ulceration. This concept is supported by the finding that duodenal ulcer patients are more likely to be infected with virulent strains than the normal population. Against this concept is the fact that the prevalence of duodenal ulceration in a country is not related to the prevalence of virulent strains. In countries where the prevalence of virulent strains is high there is no corresponding increase in the prevalence of duodenal ulceration [82].

Besides the previous difficulties there are other anomalies that cast doubt as to whether *H. pylori* infection could be the primary factor in duodenal ulceration.

(a) There are a large number of endoscopy reports from different countries of *H. pylori*-negative duodenal ulceration unrelated to NSAIDs ranging from 14% to 72% and occurring more often in countries with a low prevalence of *H. pylori* infection [68, 83–87] and in patients with a short history of ulceration [88, 89]

(b) There are several reports of duodenal ulcer recurring after eradication without reinfection [68, 84].

(c) As low as 50% of acute duodenal ulcer perforations are *H. pylori* negative [90].

Thus if the organism is not responsible for actually causing duodenal ulcer, we need to find another cause for the patients who have an ulcer whether they are infected or not.

2.1. The Role of Acid. For many years gastric acid secretion was regarded as the primary cause of duodenal ulceration. Surgical measures or medical treatment with antisecretory drugs resulted in long-term cure of the ulceration despite persistence of *H. pylori* infection.

Patients with duodenal ulcer lie in two groups related to their maximal secretory capacity: those lying above the 95% tolerance limits of the normal population and those lying within the normal limits. In each band of secretion within the normal range, the risk of developing a duodenal ulcer increases with increasing maximal acid secretion, until at greater than the 95% upper tolerance limit of the population most developed duodenal ulceration [91]. *H. pylori* infection is thought to produce an increase in acid secretion due to hypergastrinemia resulting from colonization of the antrum. However, colonization is rarely confined to the antrum, and as a result of gastritis involving the corpus it has been shown that patients with duodenal ulcer who are infected actually have a smaller maximal gastric secretion than those who are not infected [92].

Below the lower border of the normal range in nonulcer individuals there is a band of secretion in which no subject with peptic ulceration lies, fitting in with the dictum of Schwarz, "No Acid, No Ulcer" [93].

In conclusion, in duodenal ulcer patients lying within the normal range of acid secretion some other factors in addition to acid must prevail predisposing to the ulceration and this is probably related to mucosal resistance to the effect of acid.

2.2. The Role of Mucosal Resistance. At first sight it seems obvious that the *H. pylori* infection must be this additional factor affecting mucosal resistance but this would not fit in with the discrepancies noted previously, in particular that a large number of people with *H. pylori* infection in the normal range of acid secretion do not develop duodenal ulcers. (An alternative role for *H. pylori* infection is described later.)

Smoking and nonsteroidal anti-inflammatory drugs (NSAIDs) are known to be such predisposing factors. A more important and universal factor is the presence or absence of protective substances in the diet which protect the mucosa. The geographical differences in the prevalence of duodenal ulceration [94–98], which are unrelated to the prevalence of *H. pylori* infection, do bear relationship to the content of protective lipids in the staple diets (certain phospholipids and sterols) [99, 100]. These could also account for the appearance of duodenal ulceration at the beginning of the twentieth century which coincided with the introduction of roller milling resulting in the increasing refinement of wheat, maize, and rice and the removal of these protective lipids.

2.3. The Role of H. pylori Infection. It is generally assumed that *H. pylori* infection is a chronic infection, but in countries with a low prevalence of *H. pylori* infection it has been shown that it can be labile, depending on the level of acid secretion in the stomach [101]. *H. pylori* colonization is very dependent on the pH levels of the acid in the stomach. In vitro, growth of *H. pylori* is restricted to pH levels of 6.5–7.5, the optimal pH being 7.0. At pH 3.0–3.5 the organism is no

longer viable except in the presence of urea, as occurs in the stomach. *H. pylori* produces urease and this reacts with urea producing ammonia and growth can occur down to a pH of 1.5, below which the organism is not viable [102–104].Thus colonization of the stomach by *H. pylori* depends on favorable pH levels; both highly acid and highly alkaline conditions kill the organism, the range permitting growth in the stomach being between pH 1.5 and pH 7.5. At pH levels below 1.5 and above 7.5 it cannot survive.

The number of *H. pylori* negative duodenal ulcer patients in countries of low *H. pylori* prevalence is high and occurs predominantly in those with a short history of ulceration [88, 89]. It is probable that in these patients the initial level of acidity is high enough to cause the ulceration and also high enough to prevent *H. pylori* colonization. The presence of *H. pylori* in later cases may be the result of treatment and the reduction of acid secretion, permitting colonization with *H. pylori*. It is in this process of colonization that virulent strains may outperform nonvirulent ones, accounting for the observed preponderance of virulent strains in patients with duodenal ulcer.

It is noteworthy that when Marshall et al. in 1984 infected himself with *H. pylori,* he had taken a dose of ranitidine to reduce his acid secretion [105]. Two weeks later when the acid level had returned to normal the *H. pylori* infection had spontaneously disappeared.

The toxins released by *H. pylori* interfere with neoangiogenesis and with the healing of wounded duodenal epithelial cells [106, 107]. In patients with duodenal ulcer migration of tongues of antral mucosa secreting neutral mucus (duodenal gastric metaplasia) occurs in the duodenum, possibly related to distal displacement by an increased parietal cell mass. *H. pylori* cannot colonize normal duodenum in the presence of the acid mucin secreted by the goblet cells, but because of the neutral mucus secreted by antral mucosa *H. pylori* organisms from the stomach can colonize these areas of metaplasia. The toxic effect of this local colonization of the duodenum by *H. pylori* prevents the ulcer from following its natural course of healing and makes it chronic. Eradication of the infection permits the healing process to take place.

2.4. Conclusion. There is no doubt of the value of *H. pylori* eradication leading to long-term healing of duodenal ulcers, but this does not mean that the *H. pylori* infection is the initial or primary cause of the duodenal ulceration. The most important cause remains to be acid secretion, which in cases of high acid secretion may be the sole cause, but in other cases is combined with reduced mucosal resistance, in which the absence of dietary protective lipids is an important factor.

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Clinical Study

Serum Prohepcidin Levels Are Lower in Patients with Atrophic Gastritis

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Background/Aim. Hepcidin, an iron regulatory hormone, is increased in response to inflammation and some infections. We investigated the relationships among serum prohepcidin, iron status, *Helicobacter pylori* infection status, and the presence of gastric mucosal atrophy. *Methods*. Seventy subjects undergoing esophagogastroduodenoscopy underwent multiple gastric biopsies, and the possibility of *H. pylori* infection and the degree of endoscopic and histologic gastritis were investigated. In all subjects, serum prohepcidin and iron parameters were evaluated. *Results*. No correlations were observed between serum prohepcidin levels and the other markers of anemia, such as hemoglobin, serum iron, ferritin, and total iron binding capacity. Serum prohepcidin levels were not significantly different between the *H. pylori*-positive group and the *H. pylori*-negative group. Serum prohepcidin levels in atrophic gastritis patients were significantly lower than those in subjects without atrophic gastritis irrespective of *H. pylori* infection. *Conclusion*. Serum prohepcidin levels were not altered by *H. pylori* infection. Serum prohepcidin levels decrease in patients with atrophic gastritis, irrespective of *H. pylori* infection. It suggests that hepcidin may decrease due to gastric atrophy, a condition that causes a loss of hepcidin-producing parietal cells. Further investigations with a larger number of patients are necessary to substantiate this point.

1. Introduction

Human hepcidin, a 25-amino-acid peptide first identified in human urine and plasma that is secreted mainly from the liver, exerts *in vitro* antibacterial and antifungal activities [1, 2]. Prohepcidin, an 84-amino-acid precursor form of hepcidin, is found in blood [3]. Hepcidin is an acute-phase reactant, and its expression is upregulated via interleukin (IL)-6 during bacterial infection and inflammation [4]. In addition, hepcidin plays a major role in homeostatic regulation of iron metabolism. This peptide acts by binding to the cellular iron exporter ferroportin and inducing its internalization and degradation, thus trapping iron in enterocytes, macrophages, and hepatocytes [5]. Hepcidin synthesis is increased by iron overloading and decreased by iron deficiency [6, 7].

Helicobacter pylori infection with or without coexisting autoimmune gastritis has been implicated in several recent studies as an important cause of iron deficiency anemia (IDA) in patients with unexplained IDA [8]. The possible pathogenic mechanisms include occult blood loss secondary to chronic erosive gastritis, decreased iron absorption secondary to atrophy-associated gastric hypochlorhydria, and increased iron uptake and utilization by *H. pylori* [9]. Moreover, iron-deficient patients who have *H. pylori* infection seem not to respond well to oral iron therapy until the bacterium had been eradicated [10–12]. This hypothesis was confirmed by a study showing impaired absorption of iron after oral loading in infected subjects and reversion to normal after eradication [13]. It has been suggested that the reason for the failure of patients with *H. pylori* infection to respond

to iron might be the production of hepcidin or hepcidin mimetics by microorganisms [14, 15]. A recent study showed that gastric hepcidin expression was significantly upregulated in *H. pylori*-infected patients and normalized by *H. pylori* eradication [16]. The study also demonstrated that gastric hepcidin was localized in parietal cells, which regulate gastric acid production.

Serum prohepcidin concentrations are significantly decreased in patients with hereditary hemochromatosis [3], increased with declining kidney function [17], and are positively correlated with hematocrit in chronic hemodialysis patients [18]. In this study, we evaluated the relationships among serum prohepcidin, iron status, *H. pylori* infection status, and the presence of gastric mucosal atrophy.

2. Materials and Methods

2.1. Study Population. This was a single center, observational case-control study including 70 subjects who underwent routine endoscopic examination of gastrointestinal symptoms at the Uijeongbu St. Mary's Hospital between September 2005 and August 2006. Exclusion criteria were previous eradication therapy or the use of bisthmus compounds, proton pump inhibitors, antibiotics, or antisecretory drugs within the previous 2 months. Additional exclusion criteria were pregnancy or lactation, severe systemic illness, manifest clotting disorders or the use of anticoagulants, and a history of blood transfusion or iron supplement therapy.

2.2. Diagnosis of H. pylori Infection. During endoscopy, four biopsies (two from the antrum, two from the corpus) were taken. Hematoxylin and eosin (HE) staining and Giemsa staining were performed using serial sections of four specimens. The sections were independently assessed by two blinded pathologists. The ^{13}C-Urea Breath Test (UBT) was performed after an overnight fast or at least an 8 h fast. A baseline breath sample was placed into a collection tube. An aliquot of 75 mg of ^{13}C-urea dissolved in 75 mL of citric acid solution was given orally (Helikit; Isodiagnostika, Edmonton, Canada). Another breath sample was collected after 30 min. Breath samples were subsequently analyzed to determine the $^{13}C/^{12}C$ ratio by mass spectrometry (HeliView; MediChems, Seoul, Republic of Korea). The $^{13}C/^{12}C$ ratio of each breath sample was expressed as a milli-percentage (‰). Change in the ^{13}C value over baseline was expressed as delta ^{13}C. A positive result was defined as an increase of >4‰. Patients were considered to be negative for *H. pylori* if both histological examination and ^{13}C-UBT results were negative. Patients were considered to be positive for *H. pylori* if any one of the tests was positive.

2.3. Diagnosis of Atrophic Gastritis. Atrophic changes of the gastric mucosa on endoscopy were graded according to Kimura-Takemoto classification [19]. Atrophic patterns were classified into eight types by the location of the atrophic border. The C-0 pattern shows an endoscopically normal stomach without atrophic change in any area. C-1, -2, and -3 denote closed-type atrophic patterns. In the C-1 type, atrophic changes are limited to the antrum. Atrophic borders lying on the lesser curvature of the lower body define the C-2 pattern, and those on the upper body define the C-3 pattern. Meanwhile, O-1, -2, and -3 denote open-type atrophic patterns. In the O-1 type, the atrophic border is located within the lesser curvature of the body; in the O-2 type, the border is located in the anterior and posterior walls; and in the O-3 type, the border is located in the greater curvature. A histological diagnosis of atrophic gastritis was made according to the updated Sydney System using a biopsy specimen taken from the lesser curvature of the lower body [20]. Gastric mucosal inflammation (mononuclear cell infiltration), inflammatory activity (neutrophil infiltration), atrophy, and intestinal metaplasia were each assessed semi-quantitatively and graded as 0, 1, 2, or 3.

2.4. Laboratory Analysis. Blood samples were collected from all participants who had fasted overnight. Laboratory tests, including a complete blood count, total protein, albumin, hepatic and renal function tests, serum iron, total iron biding capacity (TIBC), and ferritin were performed using standard laboratory methods. Patients with a hemoglobin (Hb) of <14.0 g/dL (men) or <12.0 g/dL (women) were considered to be anemic, and ferritin concentrations of <30 μg/L (men) or <13 μg/L (women) were considered indicative of iron deficiency.

The serum prohepcidin level was measured using the DRG Diagnostics Hepcidin Prohormone enzyme-linked immunosorbent assay (ELISA) (DRG Instruments GmbH, Marburg, Germany), according to the manufacturer's instructions. The employed antibody detects both the proregion and prohepcidin (aa 25–84). The sensitivity of the assay was 3.95 ng/mL, intra-assay coefficient of variation (CV) was 4.69%, and inter-assay CV was 4.82%.

2.5. Statistical Analysis. Values are expressed as mean ± standard deviation (SD). Continuous data were compared using an independent-samples t-test, whereas the categorical data were analyzed using χ^2 or Fisher's exact tests. For correlation analysis, the Spearman nonparametric correlation was used. Data were processed and analyzed with SPSS, version 12.0 (Chicago, IL, USA). A value of $P < 0.05$ was considered to indicate statistical significance for all tests.

2.6. Ethics Statement. This study protocol was approved by the Institutional Research Ethics Board of Uijeongbu St. Mary's Hospital of the Catholic University of Korea (IRB No. UCMC06BR006) and adhered to the Declaration of Helsinki. All of the study subjects completed an informed consent form before participating in the study. The informed consent was confirmed by the board.

3. Results

A total of 70 patients with gastrointestinal symptoms (29 men, 31 women; mean age, 41.7 years; range, 21–77 years) were included in the study. When the patients were separated into *H. pylori*-positive ($n = 35$) and *H. pylori*-negative

Table 1: Demographic feature, hematological and biochemical parameters in the study groups.

	Atrophic gastritis-positive ($n = 35$)	Atrophic gastritis-negative ($n = 35$)	P value
Male gender	$n = 17$ (48.6%)	$n = 12$ (34.3%)	0.231
Age	45.9 ± 10.6	37.4 ± 11.9	0.002
H. pylori positivity	$n = 25$ (71.4%)	$n = 10$ (28.6%)	0.000
Hb, g/dL	14.0 ± 1.72	13.0 ± 2.55	0.062
Serum iron, μg/dL	113.0 ± 42.49	91.9 ± 55.87	0.080
Serum ferritin, μg/L	111.74 ± 136.58	83.24 ± 104.50	0.331
TIBC, μmol/L	303.7 ± 40.56	331.46 ± 63.29	0.033
Serum prohepcidin, ng/mL	225.2 ± 47.84	257.6 ± 64.40	0.020

Table 2: Correlation between the serum prohepcidin and the parameters for anemia in patients with and without atrophic gastritis.

	Atrophic gastritis-positive ($n = 35$)		Atrophic gastritis-negative ($n = 35$)	
	Correlation coefficient (r)	P-value	Correlation coefficient (r)	P-value
Prohepcidin X Hb	0.036	0.837	0.267	0.120
Prohepcidin X SI	−0.200	0.250	0.026	0.880
Prohepcidin X SF	0.224	0.195	0.197	0.256
Prohepcidin X TIBC	0.171	0.327	0.104	0.552

R: Spearman coefficient; Hb: hemoglobin; SI: serum iron; SF: serum ferritin; TIBC: total iron binding capacity.

($n = 35$) groups, no significant differences in the hematological or biochemical parameters were observed between the two groups. However, a difference in atrophic gastritis was identified, which was significantly more frequent ($P < 0.001$) in the *H. pylori*-positive group than in *H. pylori*-negative group (71.4% versus 28.6%, resp.). Anemia was diagnosed in nine (12.9%) patients, three of whom were *H. pylori*-positive and six of whom were *H. pylori*-negative. Among anemic patients, only three in the *H. pylori*-negative group had iron deficiency. The mean serum prohepcidin level was 241.4 ng/mL (SD, 58.6; range, 137–401) among all subjects, 238.8 ng/mL (SD, 60.9; range, 144–401) in the *H. pylori*-positive group, and 244.1 ng/mL (SD, 57.0; range, 137–359) in the *H. pylori*-negative group. The serum prohepcidin level correlated with the hemoglobin ($r = 0.355$, $P = 0.036$) and ferritin ($r = 0.371$, $P = 0.028$) levels in the *H. pylori*-positive group. No correlation was observed between the serum prohepcidin level and the other markers of anemia, such as hemoglobin, serum iron, ferritin, and TIBC, in the *H. pylori*-negative group.

Twenty-five *H. pylori*-negative subjects showed an endoscopically normal stomach with a C-0 atrophic border and histologically normal fundic mucosa, and 10 subjects showed mild atrophic change (C-1). Ten *H. pylori*-positive subjects showed no atrophic changes and histologically normal fundic mucosa, and 25 subjects showed various endoscopic atrophic changes (C-1, $n = 14$; C-2, $n = 4$; and C-3, $n = 7$). The descriptive statistics are reported in Table 1. Patients with atrophic gastritis were older than subjects without atrophic gastritis. However, there was no correlation between the age and the serum prohepcidin level among all subjects. Serum prohepcidin levels were significantly lower in atrophic gastritis patients than in subjects without atrophic gastritis (225.2 ± 47.8 versus 257.6 ± 64.4 ng/mL, $P = 0.020$) irrespective of *H. pylori* infection (Figure 1). However, in atrophic gastritis patients, the degree of atrophy was not correlated to the serum prohepcidin level ($P = 0.058$). No correlation was observed between the serum prohepcidin level and the other markers of anemia, such as hemoglobin, serum iron, ferritin, and TIBC, in patients with and without atrophic gastritis (Table 2).

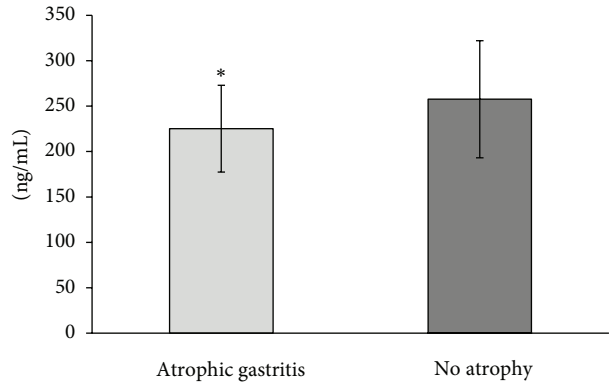

Figure 1: Serum prohepcidin levels in 35 atrophic gastritis patients and in 35 subjects without atrophic gastritis.

4. Discussion

Since its discovery, hepcidin has attracted the attention of investigators because of its ability to regulate iron metabolism and exert antimicrobial activity against numerous bacteria and fungi [1, 2, 4–7]. In the present study, the serum level of prohepcidin, a hepcidin precursor, and its relationship with iron metabolism and *H. pylori* infection were evaluated. There was no relationship between prohepcidin, iron deficiency parameters, and *H. pylori* infection.

Hepcidin plays a major role in the iron regulatory mechanism through inhibition of iron export from enterocytes, macrophages, and hepatocytes [5]. Hepcidin levels increase in response to iron loading, reducing intestinal iron absorption and inhibiting iron release from stores [21]. Meanwhile, iron deficiency produces low hepcidin levels, resulting in enhanced iron absorption and iron mobilization from stores. In addition, hepcidin is induced by inflammation, causing its sequestration in stores [7]. The resulting iron decrease contributes to anemia in chronic disease. The relationship of hepcidin to disorders of iron metabolism has been established via the measurement of urinary hepcidin concentrations using immune-dot [7], sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), western blot [4], and surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) [22]. Recent studies reported two types of hepcidin assays for the semiquantitative or quantitative determination in human serum. First, mass spectrometric assays detect the characteristic mass of the active 25-amino-acid hepcidin species or its fragments [23–25]. However, these assays require access to specialized equipment and are not widely available. Second, an ELISA specific for the refolded, mature 25-amino-acid form was developed [26]. The serum hepcidin level by ELISA was inversely correlated with iron absorption from supplemental and food-based nonheme iron sources in iron-replete healthy women [27]. However, subsequent evaluation is required to prove the usefulness of this method.

In the present study, we measured serum prohepcidin levels; this is one of the limitations of this study. Prohepcidin is far more immunogenic than hepcidin, and a prohepcidin ELISA is commercially available. Our study failed to show any association between serum prohepcidin concentrations and iron deficiency parameters. These results are in agreement with those of previous studies in which the serum prohepcidin concentration was correlated poorly with markers of iron homeostasis, such as intestinal iron absorption [27–29]. In addition, a relationship between serum concentrations of prohepcidin and those of hepcidin 25 was not found [27]. However, another study reported a significant positive correlation between prohepcidin and hepcidin serum levels [30].

An association between *H. pylori* infection and IDA has been reported [8–12]. Our study showed that anemia was not increased in subjects with *H. pylori* infection and showed no relationship with hepcidin. This may be due to the small sample size, the fact that the study cohort was fairly uniform, and the fact that most subjects had normal iron stores. Nevertheless, our data do not support the proposal that hepcidin plays a key role in the primary mechanism of *H. pylori*-induced anemia. Previous studies have demonstrated that hepcidin and prohepcidin serum levels by ELISA were not altered by *H. pylori* infection or eradication even when hepcidin was detected in human gastric juice [16, 31, 32]. These findings suggest that hepcidin may exert local rather than systemic functions.

Schwarz et al. recently reported a new role for hepcidin in the stomach [16]. In this study, quantitative RT-PCR demonstrated abundant hepcidin expression in the fundus/corpus part of the glandular stomach in mice, rats, and humans. Hepcidin was localized in gastric parietal cells by immunofluorescence staining and *in situ* hybridization. Gastric hepcidin expression in patients and in AGS cells was significantly upregulated during *H. pylori* infection. In addition, *H. pylori* eradication resulted in normalization of hepcidin expression levels. Moreover, hepcidin-knockout mice displayed decreased H^+/K^+-ATPase gene expression, significant bacterial overgrowth, and reduced gastric gene expression. These findings suggest that hepcidin regulates gastric acid production and may contribute to the development of gastric ulcers. In the present study, atrophic gastritis was found to be present in 50%. In Korea, the seroprevalence of *H. pylori* was high (59.6%) in the Korean population among asymptomatic Korean adults in 2005 [33]. In addition, the prevalence of atrophic gastritis in the antrum and body was 42.5% and 20.1%, respectively, in Korean population without significant gastroduodenal disease [34]. We found that serum prohepcidin levels decreased in subjects with gastric atrophy, irrespective of *H. pylori* infection. This finding might be explained by the loss of hepcidin-producing cells caused by gastric atrophy. However, we did not find that serum prohepcidin levels are related to the degree of atrophy. In addition, correlation between the serum prohepcidin and the parameters for anemia was not found in patient with atrophic gastritis. This can be explained by the small number of patients enrolled and by the lack of evaluation of hepcidin expression in gastric tissues. A recent study demonstrated that gastric hepcidin expression decreased in hypergastrinemic ING-mice with chronic gastric *H. pylori* infection and resulted in the upregulation of the expression of various downstream iron absorption and efflux genes such as Ferroportin 1, Divalent metal transporter 1, and Transferrin receptor 1 [35]. These findings suggest that the decrease of gastric hepcidin expression due to the loss of hepcidin-producing parietal cells may function as an iron regulator. However, further studies regarding the functional role of hepcidin and iron transporter in the gastric mucosa are required because iron is mainly absorbed in the small intestine.

Pepsinogen, an aspartic proteinase secreted mainly by gastric cells, is classified immunologically as pepsinogen I (PG I) and pepsinogen II (PG II). Whereas PG I is secreted only from the gastric fundic mucosa, PG II is secreted from the cardiac, fundic, and antral mucosae of the stomach [36]. The effects of gastric atrophy on serum PG concentrations are lower PG I and stable or increased PG II levels, and this results in a lower PG I/II ratio [37]. Gastrin is another valid tool for detection of gastric body mucosal atrophy, and increased serum gastrin is a regular feature of atrophic body gastritis due to the loss of negative feedback by gastric acidity [38]. Further study is needed to evaluate the relationships between hepcidin and gastric mucosal atrophy using the gastrin and/or pepsinogen I/II ratio.

In conclusion, our data suggest that serum prohepcidin levels were not altered by *H. pylori* infection. Serum prohepcidin levels decreased in patients with atrophic gastritis, irrespective of *H. pylori* infection. It suggests that hepcidin may decrease due to gastric atrophy, a condition that causes a

loss of hepcidin-producing parietal cells. Further studies with a larger number of atrophic gastritis patients are necessary to better investigate the relationship between hepcidin and atrophic gastritis.

Conflict of Interests

All authors have no conflict of interests to declare.

Authors' Contribution

H.-K. Kim and E.-C. Jang contributed equally to this study.

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Clinical Study

Association of IS605 and *cag*-PAI of *Helicobacter pylori* Isolated from Patients with Gastrointestinal Diseases in Taiwan

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Background. The *cag* pathogenicity island (*cag*-PAI) is one of the most important virulent determinants of *Helicobacter pylori*. An insertion sequence (IS) element of *cag*-PAI (IS605) has been found to generate *H. pylori* strains with varying virulence. *Aim*. To evaluate the impact of IS605 and *cag*-PAI on *H. pylori* strains isolated from Taiwanese patients with severity of gastric diseases. *Methods*. *H. pylori* isolates were cultured from gastric biopsies from 99 patients with peptic ulcer, chronic gastritis, and gastric carcinoma. Six distinct, well-separated colonies were isolated from each patient and analyzed by genotyping. *Results*. *cagA*, *cagE*, *cagM*, *cagT*, *orf*10, and *orf*13 were found to be present in 90.0%–100.0% of the *H. pylori* isolates. A total deletion of *cagA*, *cagE*, *cagM*, *cagT*, *orf*10, and *orf*13 was found in 1 isolate (1.0%). The IS605 element was found to be positive in 15.2% of the isolates. The presence of IS605 was higher in *H. pylori* isolated from patients with gastric carcinoma (25.0%) than in patients with duodenal ulcer (6.5%) or chronic gastritis (6.3%) ($P < 0.001$). *Conclusions*. The majority of the patients examined had intact *cag*-PAI. IS605 was present in 15.2% and was higher in *H. pylori* isolated from patients with gastric carcinoma than in those with peptic ulcer.

1. Introduction

Infection with *Helicobacter pylori* is one of the most common bacterial infections in humans [1]. It has been closely linked with chronic gastritis, peptic ulcer, gastric carcinoma, and mucosa-associated lymphoid tissue (MALT) lymphoma [2]. The *cag* pathogenicity island (*cag*-PAI), a major determinant of *H. pylori* virulence, has been well studied [3, 4]. *cag*-PAI is an ~40-kb region in the *H. pylori* genome that contains a cluster of around 30 genes, including a type IV secretion system (T4SS) and the cytotoxin-associated gene A (CagA) [5]. Translocation of CagA into gastric epithelial cells requires delivery by T4SS and subsequent induction of cell signaling, which contributes to the development of pathogenesis in gastric mucosa [6].

An insertion sequence (IS) element, IS605, found in some strains disrupts an otherwise-uninterrupted *cag*-PAI unit, thereby splitting it into 2 regions (Figure 1). These regions, termed *cag* I and *cag* II, contain at least 14 and 16 open reading frames (*orf*s), respectively [7]. Insertion of IS605 between *cag* I and *cag* II generates *H. pylori* strains with different levels of virulence [8, 9]. Censini et al. reported that the *cagA* gene is closely linked to the intact *cag*-PAI [4]. However, the presence of the *cagA* gene does not guarantee the existence of an intact *cag*-PAI [10–12].

Diversity within *cag*-PAI is noted between the people belonging to the Eastern and Western hemispheres [13]. Only one-half to two-thirds of the isolates from the western world carry *cag*-PAI. In contrast, nearly all East Asian isolates carry *cag*-PAI [14, 15]. Therefore, *cagA* and other genes comprising the *cag*-PAI should be individually investigated. Moreover, *cag*-PAI appears to be disrupted in the majority of isolates globally [14]. Partial deletion of *cag*-PAI has been reported in 4%–88% isolates [12–14, 16]. However, the clinical relevance

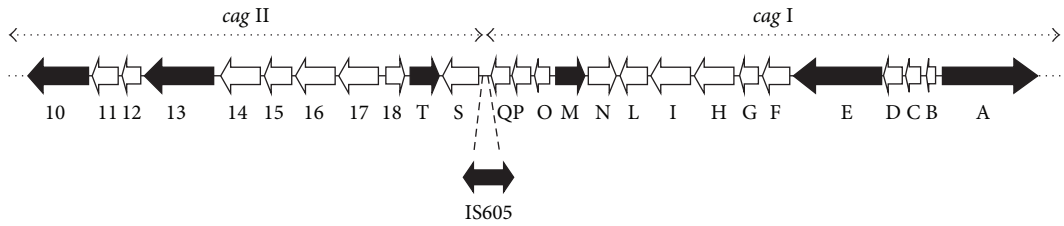

Figure 1: Structure of *H. pylori cag* pathogenicity island. The localizations of *cag* I, *cag* II, and IS605 are shown. The genes assessed in this study including *cagA*, *cagE*, *cagM*, *cagT*, *orf*10, and *orf*13 are indicated as close black.

of strains with an intact *cag*-PAI remains controversial [17]. The aim of this study was to evaluate the impact of IS605 and *cag*-PAI on *H. pylori* strains isolated from Taiwanese patients with respect to disease severity.

2. Materials and Methods

2.1. Study Subjects. The study between January 2001 and September 2009 comprised patients who were examined for gastric ulcer, duodenal ulcer, chronic gastritis, or gastric carcinoma, as well as those who were clinically diagnosed with upper gastrointestinal problems. All had completed a self-administered questionnaire prior to being enrolled in the study. Patients were excluded from the investigation if they presented with any of the following: inability to give written informed consent, bleeding tendency (platelet count $<50{,}000/mm^3$, prothrombin time 3 seconds more than controls, if on anticoagulants), or having taken H_2-receptor antagonists or proton pump inhibitors within 2 weeks of enrollment. The study was approved by the Clinical Research Committee of the Veterans General Hospital, Taipei, Taiwan.

2.2. H. pylori Strains and Bacterial Culture. H. pylori isolates were cultured from gastric biopsy specimens and were identified by their positive reactivity for catalase, urease, and oxidase activities [18]. The isolates were cultured at 37°C on brain heart infusion (BHI) agar plates supplemented with 7% horse blood (containing nalidixic acid 10 μg/mL; trimethoprim 5 μg/mL, vancomycin 3 μg/mL, and amphotericin 2 μg/mL) under 12% CO_2 with high humidity as in our previously reported study [19]. The rapid urease test was performed using an in-house urease test at room temperature for color change up to 24 hours. The test was defined as positive if the color changed from yellow to red [19, 20].

2.3. Polymerase Chain Reaction. After obtaining positive cultures from the biopsy specimens, 6 isolated colonies from a single culture plate were tested for genotypes with polymerase chain reaction (PCR). These colonies were homogenized in guanidinium isothiocyanate, using a sterile micropestle. DNA were extracted, washed, and eluted in 100 μL of 10 mM Tris-Hcl (pH 8.3). Two microliters of the purified DNA was used for each PCR reaction. Eight primers were employed to assess the upstream and downstream of *cag*-PAI and IS605: *cagA*, *cagE*, *cagM*, *cagT*, *orf*10, *orf*13, and IS605 (*tnpA* and *tnpB*) (Table 1 and Figure 1). PCR was performed under the following conditions: 30 cycles at 94°C for 1 min, at 50.9°C–63°C for 2 min, and at 72°C for 1 min, and a final extension at 72°C for 5 min. PCR products were analyzed on 1.0% agarose gels. Mixed infection was defined if there was different *cagA*, *cagE*, *cagT*, *cagM*, and *orf*10, *orf*13 among the 6 isolates from one plate.

2.4. Statistical Analysis. Descriptive statistics are reported as the proportion for categorical variables with 95% confidence intervals (CI), and means ± standard deviation for continuous variables. The CIs for all proportions are calculated using the standard approximation of binomial. The Chi-square test with Fisher's exact test was used to compare the clinical variables and results. A P value of less than 0.01 was considered significant.

3. Results

Between January 2001 and September 2009, 449 patients (37 patients with chronic gastritis, 101 had duodenal ulcer, 140 with gastric ulcer, and 171 with gastric carcinoma) were enrolled in this study underwent endoscopic examination and *H. pylori* culture studies. Of the enrolled subjects, 234 patients were positive for *H. pylori* infection: 25 (68%) with chronic gastritis, 66 (65%) with duodenal ulcer, 77 (55%) with gastric ulcer, and 66 (39%) with gastric carcinoma. Among those patients, we randomly selected 99 subjects (16 patients with chronic gastritis, 31 with duodenal ulcer, 32 with gastric ulcer, and 20 with gastric carcinoma) for further analysis of their *H. pylori* isolates. Six distinct, well-separated colonies were obtained from each patient making a total of 594 isolates for *H. pylori* genotyping. In the 99 subjects, mixed infections of *H. pylori* strains were found in 11 (11.1%) patients.

Our data showed that *cagA*, *cagE*, *cagM*, *cagT*, *orf*10, and *orf*13 were present in between 90.0% and 100% of the isolates (Table 2). There were no differences in the *cag*-PAI status among isolates from patients with various clinical outcomes. Total deletion of *cagA*, *cagE*, *cagM*, *cagT*, *orf*10, and *orf*13 was found in 1 (1.0%) isolate from a patient with gastric carcinoma. There was only 1 isolate from patient with gastric ulcer had *cagA* deletion. Deletion of *cagM*, *cagT*, *orf*10, and *orf*13 was found in 2 (2.0%) isolates (1 isolate from patient with gastric carcinoma and 1 isolate with gastric ulcer), and *cagE* deletion was found in 4 (4.0%) isolates (each isolated from patient with gastric carcinoma, gastric ulcer, duodenal ulcer, or chronic gastritis, resp.).

Table 1: PCR primers used to detect *CagA, CagE, CagM*, *CagT, orf10, orf13, TnpA*, and *TnpB* in *H. pylori* isolates.

Genes	Primers	Nucleotide sequences (5′–3′)	Length of PCR products	Location in genome of reference[†]
cagA	cagA-F	GATAACAGGCAAGCTTTTGAGG	349	14994–15342
	cagA-R	CTGCAAAAGATTGTTTGGCAGA		
cagE	cagE-F	GTTACATCAAAAATAAAAGGAAGCG	735	12342–13076
	cagE-R	CAATAATTTTGAAGAGTTTCAAAGC		
cagM	cagM-F	ACAAATACAAAAAAGAAAAAGAGGC	587	4815–9264
	cagM-R	ATTTTTCAACAAGTTAGAAAAAGCC		
cagT	cagT-F	TCTAAAAAGATTACGCTCATAGGCG	490	1182–1671
	cagT-R	CTTTGGCTTGCATGTTCAAGTTGCC		
*orf*10	*orf*10-F	AATAGTGCTTTCTTTAGGATTAGCG	658	8970–9627
	*orf*10-R	CCGATTTAATCCTTTCGCTTATGTG		
*orf*13	*orf*13-F	CGTTCATGTTCCATACATCTTTGGC	617	704–1320
	*orf*13-R	GATTTATAGCGATCTAAGAAACCGC		
tnpA	tnpA-F	ATCAGTCCAAAAAGTTTTTTCTTTCC	338	154–541
	tnpA-R	TAAGGGGGTATATTTCAACCAACCG		
tnpB	tnpB-F	CGCTCTCCCTAAATTCAAAGAGGGC	578	954–1531
	tnpB-R	AGCTAGGGAAAAATCTGTCTATGCC		

[†]GenBank accession number. AF282853.1.

Table 2: Prevalence of *H. pylori* virulence factors among isolates from patients with gastric carcinoma, gastric ulcer, duodenal ulcer, and chronic gastritis.

Diagnosis[†]	GC	GU	DU	CG	Total
Number of isolates	20	32	31	16	99
CagA	20 (100.0%)	31 (96.8%)	31 (100.0%)	16 (100.0%)	98 (99.0%)
CagE	19 (95.0%)	31 (96.9%)	30 (96.8%)	15 (93.8%)	95 (96.0%)
CagM	19 (95.0%)	31 (96.8%)	31 (100.0%)	15 (93.8%)	96 (97.0%)
CagT	19 (95.0%)	31 (96.8%)	31 (100.0%)	16 (100.0%)	97 (98.0%)
*orf*10	18 (90.0%)	32 (100.0%)	31 (100.0%)	16 (100.0%)	97 (98.0%)
*orf*13	18 (90.0%)	32 (100.0%)	31 (100.0%)	16 (100.0%)	97 (98.0%)

[†]GC: gastric carcinoma; GU: gastric ulcer; DU: duodenal ulcer; CG: chronic gastritis.

IS605 was found to be present in 15.2% (15/99) of the isolates in this study (Table 3). The presence of IS605 was significantly higher in the *H. pylori* strains isolated from patients with gastric carcinoma (5/20, 25.0%) than in patients with duodenal ulcer (2/31, 6.5%) or chronic gastritis (1/16, 6.3%) ($P < 0.001$). It was also higher in isolates from patients with gastric ulcer (7/32, 21.9%) compared to those with duodenal ulcer and chronic gastritis ($P < 0.01$).

Table 3: Prevalence of IS605 in *H. pylori* isolated from patients with gastric carcinoma, gastric ulcer, duodenal ulcer, and chronic gastritis.

Diagnosis[†]	Number (%) of IS605 positive isolates
GC ($n = 20$)	5 (25.0)[*]
GU ($n = 32$)	7 (21.9)[#]
DU ($n = 31$)	2 (6.5)
CG ($n = 16$)	1 (6.3)
Total ($n = 99$)	15 (15.2)

[†]GC: gastric carcinoma; GU: gastric ulcer; DU: duodenal ulcer; CG: chronic gastritis.
[*]$P < 0.001$, GC versus DU; GC versus CG.
[#]$P < 0.01$, GU versus DU; GU versus CG.

4. Discussion

The total number of isolates used in this study was 594, and *cag*-PAI was found to be present in more than 96% of isolates, which is similar to that reported previously for eastern populations [12, 21–23]. Previous studies from the USA reported that *cagA* and *cagE* were detected in 66% and 62% *H. pylori* strains, respectively [24]. In other studies, *cagA*, *cagE*, *cagM*, and *cagT* were detected in 93%–100% of Korean and 80%–82% of the Colombian populations [13]. The presence of *cagA* and *cagE* in *H. pylori* isolated from Chinese, Indian, and Malay patients in Singapore ranged from 92.3% to 100% [12].

In southern Taiwan, *cagA*, *cagE*, and *cagT* were previously found to be present in 100% of the domestic strains [21]. Our present study of *H. pylori* isolates from patients in northern Taiwan yielded similar results, with *cagA*, *cagE*, *cagM*, and *cagT* being present in more than 96% of the isolates tested.

Patients with intact *cag*-PAI are thought to be associated with more severe clinical outcomes [10, 25]. Maeda et al. from Japan reported that strains with partial deletions within the *cag*-PAI were only derived from patients with nonulcer dyspepsia, whereas strains with an intact *cag*-PAI originated only from patients with gastric carcinoma [11]. Jenks et al. reported that intact *cag*-PAI was found in 85% and 53% of duodenal ulcer and nonulcer dyspepsia isolates, respectively, [10]. They indicated that the clinical outcome of *H. pylori* infection was not reliably predicted by any gene of the *cag*-PAI [10]. However, since the deletion of *cag*-PAI has been observed in patients with peptic ulcer and nonulcer dyspepsia, the pathogenicity of *H. pylori* may therefore not be determined by *cag*-PAI, as suggested by Kawamura et al. [25].

IS605 is located between *cag* I and *cag* II in the prototype *H. pylori* and is thought to be closely related to *cag*-PAI [4]. However, a recent study has suggested that IS605 is not related to the *cag*-PAI status [26]. In a previous study involving the Taiwanese population, all isolates were reported as positive for the *cag*-PAI, but only 36% of these isolates carried an IS605 insertion [21]. The previous controversial study [21] may have used pooled cultures for the analysis, and the actual positive rate of IS605 may be lower because of the possibility of mixed infections.

Owen et al. reported that the distribution of genomic IS605 inserts varied widely with respect to patient disease severity [27]. In this study, IS605 was detected at an overall frequency of 15.2% of isolates and in 25.0% of isolates from patients with gastric carcinoma (Table 3). Thus the presence of IS605 was found to be higher in *H. pylori* from patients with gastric carcinoma. It was also higher in isolates from patients with gastric ulcer (21.9%) compared to those with duodenal ulcer (6.5%) and chronic gastritis (6.3%). These results were consistent with previous findings by Deguchi et al. who indicated that the presence of IS605 was more frequently associated with the *cag13* gene in gastric cancer patients [9]. Kersulyte et al. report that the IS605 element family has homology at the protein level with *orfB* (putative transposase gene) [28]; this gene is present in a *Salmonella* prophage and contributes to virulence during bacterial infection [29]. This evidence suggests that the presence of the IS605 element may have a possible modifying role with respect to strain pathogenicity [4]. However, the function of IS605 in relation to *H. pylori cag*-PAI and the severity of gastric diseases still remain unclear. Further studies are required to clarify the role of IS605 in the development of gastric carcinoma.

In conclusion, a majority of patients infected with *H. pylori* contained intact *cag*-PAI. However, the presence of the *cagA* gene does not guarantee the presence of intact *cag*-PAI. The presence of IS605 is significantly higher in isolates from patients with gastric carcinoma compared to those with duodenal ulcer and chronic gastritis.

Authors' Contribution

C.-L. Perng is the co-first author.

Conflict of Interests

All authors have no conflict of interests to declare.

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Clinical Study

PCR-Based Detection and Genotyping of *Helicobacter pylori* in Endoscopic Biopsy Samples from Brazilian Patients

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Helicobacter pylori (*H. pylori*) is considered the second most prevalent infection in man. A precise diagnosis is important for treating patients with the indicative gastrointestinal symptoms. The present study analyzes the effectiveness of a molecular biology method (PCR) comparing the results obtained with the histology and with the rapid urease tests. PCR was used in the detection and genotyping of the *H. pylori* urease-C gene and the patterns which were obtained from the patients studied. 141 biopsy samples from 131 patients were evaluated. 59 paraffin biopsies samples were positive for *H. pylori* according to the histological examination. Of those, 59/12 (20.3%) were amplified using PCR. Of the 82 samples from the fresh biopsies, 64 were positive for *H. pylori* according to the rapid urease test (78%); there was an agreement of 100% with PCR. Sixty positive *H. pylori* samples were genotyped (58 samples of fresh biopsies and 2 samples of paraffin biopsies) using two restriction enzymes. The patterns observed were analyzed with the computational program BIO 1D; 11 patterns with the enzyme *HhaI* and 12 patterns with the enzyme *MboI* were found. However, it was not possible to find a statistically significant correlation between the specific genotypes and digestive pathologies. Accordingly, future research should be performed to confirm a statistically significant relationship between genotyping and gastrointestinal symptoms.

1. Introduction

The *Helicobacter pylori* (*H. pylori*) infection is currently endemic worldwide with high prevalence (up to 60%) in developing regions such as South America. The infection causes chronic Gastritis, gastric and duodenal ulcers, gastric adenocarcinoma, and mucosa-associated lymphoid tissue [1–6]. *H. pylori* is associated with several autoimmune diseases, including idiopathic thrombocytopenic purpura (ITP), Sjögren syndrome, systemic sclerosis [7], Graves' disease [8], and autoimmune pancreatitis [9]. As a result of this association with autoimmune diseases, we hypothesized that *H. pylori* might induce systemic immunological changes. Although the seroprevalence of *H. pylori* may be high in the normal population, a minority develops peptic ulcers [10, 11]. Some possibilities could justify this data: genetic differences in the host's environmental factors and bacterial strains.

A variety of tests are now available to diagnose *H. pylori* infection. Histological examination of gastric tissue, bacterial cultures, rapid urease test, use of DNA probes, and PCR analysis, when used to test gastric tissue, all require endoscopy. In contrast, breath tests, serology, gastric juice PCR, and urinary excretion of N^{15} ammonia are noninvasive tests that do not require endoscopy. PCR offers high sensitivity and specificity as a technique for the detection of *H. pylori* although the accuracy of such techniques varies widely [12]. The aim of this work is to analyze the effectiveness of the molecular biology method PCR in the detection of *H. pylori* in patients with

Table 1: Patient characteristics.

	Total (n = 141)
Patients	
Sex (male/female)	81/50
Age years (median)	48 years (range 4–90)
Disease	
Gastritis	99 (70.2%)
Ulcers	29 (20.6%)
Gastritis + ulcers	5 (3.5%)
Esophagitis	3 (2%)
Other*	5 (3.5%)

* Inflammation, duodenitis, and splenomegaly.

gastrointestinal symptoms, comparing the results with the histology and the rapid urease test and using the PCR-RFLP technique to detect *H. pylori* subtypes in endoscopic biopsy samples obtained from Brazilian patients.

2. Material and Methods

2.1. Patients. 141 samples were collected from 131 patients with several diagnoses of gastrointestinal pathologies. Among them, 99 patients who were involved in this study had Gastritis, 29 had ulcers, 5 had Gastritis and ulcers, 3 had esophagitis, and 5 had other gastrointestinal diseases. The patients were 48 years old on average; their ages varied from 4 to 90 years old. 81 were males and 50 females (Table 1). All patients were submitted to an endoscopy at the Gastrocentro (Center of Digestive Tract Studies), University Hospital, State University of Campinas, SP, Brazil, after informed consent was obtained and protocol approved by the Hospital's Ethics Committee.

2.2. Methods. The methods used for the detection of *H. pylori* were polymerase chain reaction (PCR) and PCR-RFLP for genotyping. They were chosen in order to detect the bacterium and its subtypes in endoscopic biopsies of fresh tissues and paraffin tissues. The fresh biopsy samples were conserved in physiologic serum 0.9% until the DNA was extracted. At least two, 5 to 10 mm, ribbons of paraffin were collected from the paraffin tissue. In the fresh biopsies, at least two fragments were collected.

2.2.1. DNA Extraction—Gastric Paraffin Biopsy. DNA extraction from the endoscopic biopsies fastened in paraffin followed the method described by Davis et al., 1995 [13], with some modifications.

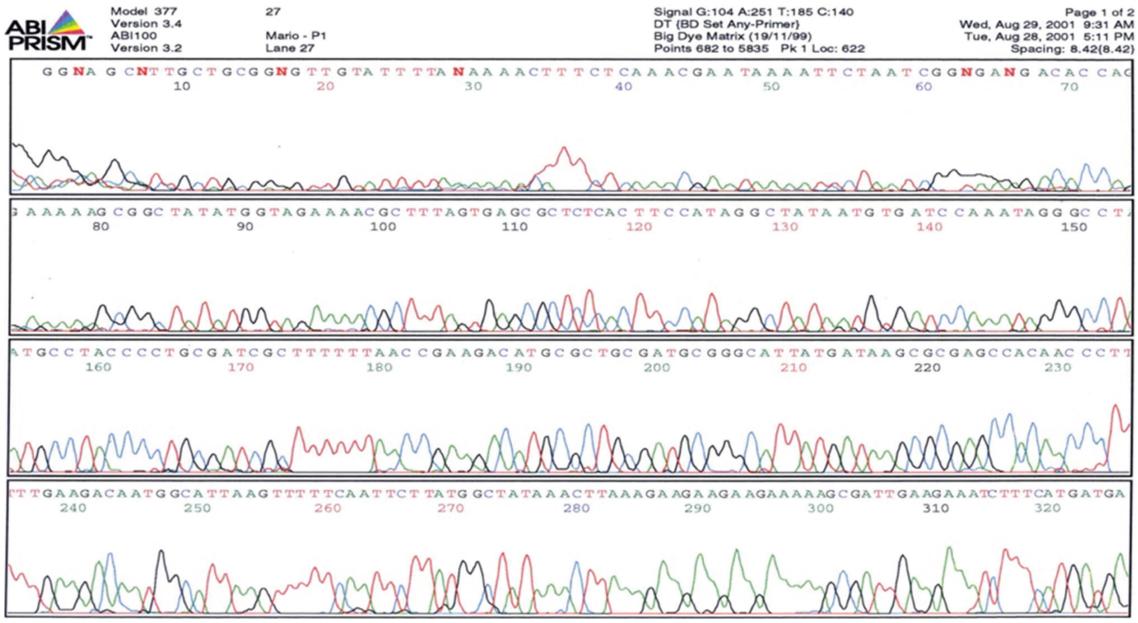
At least two, 5 to 10 mm, ribbons of paraffin were placed in a 1.5 mL Eppendorf tube. One mL of xylene was added to the samples. They were shaken, allowed to rest for 3 to 5 minutes, and then centrifuged for 5 minutes, discarding the xylene afterwards. After three washes in 100%, 95%, and 70% ethanol, respectively, the samples were dried at room temperature. Next, the material was resuspended in a solution of Proteinase K, 50 mM Tris, 0.5% SDS, and sterile water. 430 μL of phenol were added to the sample, which was homogenized and centrifuged for another 30 minutes at 14,000 RPM. The supernatant containing DNA was transferred to a new tube and 430 μL of phenol/chloroform (1 : 1) were added and centrifuged again for 5 minutes at 14,000 RPM. Chloroform/isoamyl ethanol was added (24 : 1) to the supernatant, which was homogenized and centrifuged for another 30 minutes at 14,000 RPM. After the addition of 75 μL of ammonium acetate and 750 μL of 100%, ethanol samples were inverted several times and incubated overnight to −20°C. After centrifugation for 30 minutes at 12,000 RPM to −4°C, the supernatant was discarded. The precipitate was carefully washed with 500 μL of chilled 70% ethanol, which was immediately discarded. The material was dried at room temperature and resuspended in a solution containing 50 mL of sterile water, 10 M of Tris (pH 8.0), and 1 mL of EDTA and stored at −20°C until its use.

2.2.2. DNA Extraction: Fresh Biopsy. Firstly, a fresh 3 to 7 mm biopsy section was placed in a 1.5 mL sterile tube with 190 μL of a solution that contained 0.1 M of Tris HCl (pH 7.5) and 1% of SDS. Secondly, 10 μL of proteinase K were added (10 mg/mL) to the solution. The sample was macerated and incubated overnight at 55°C. After that, 200 μL of phenol and 200 μL of both chloroform and isoamyl alcohol (24 : 1) were added. The solution was then homogenized and centrifuged for one minute. Next, the supernatant was removed, and 200 μL of chloroform/isoamyl alcohol were added, homogenized, and centrifuged for 1 minute. Next, the supernatant was removed again, and 25 μL of sodium acetate 3 M and 900 μL of 100% ethanol at −20°C were added; after vortexing the mixture, it was incubated for 30 minutes at −70°C. The samples were centrifuged for 15 minutes at 15,000 RPM. The supernatant was discarded. Lastly, the DNA was resuspended in 25 μL of distilled and sterile water. [14].

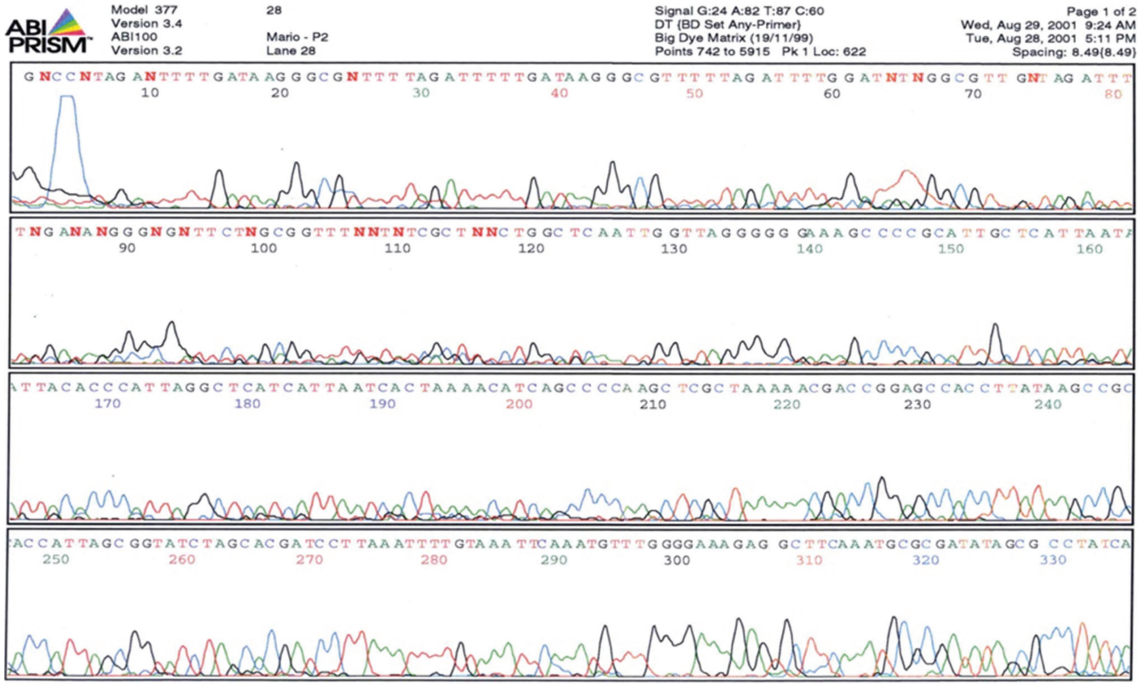
2.2.3. PCR Amplification of the H. pylori. The polymerase chain reaction followed the method described by Saiki and col. [15], with some modifications.

For each amplification reaction, 0.5 to 0.7 μL of the DNA under investigation were used, for a total reaction volume of 20.0 μL. The reaction buffer contained 50 mM KCL, 10 mM Tris-HCL pH 8.4, 2.5 mM $MgCL_2$, 2.0 pmol of each "primer," 200 μM of each deoxynucleotide triphosphate (dATP, dCTP, dGTP, and dTTP), 2.5 units of Taq polymerase (Gibco-BRL), and sufficient water to give the total volume of 20.0 μL. The reaction mixture was covered with 100 μL of mineral oil and the tubes were placed in a DNA Thermal Cycler (Perkin-Elmer).

The reactions that followed were found to be optimal. The samples were heated to 94°C for 60 s to denature the DNA, cooled to 57°C for 90 s to allow the primers and the DNA to reanneal, and then heated to 72°C for 120 s for primer extension. By the final cycle, the extension period was 7 min. A total of 40 cycles were performed. The amplified product was detected by direct gel analysis. 5 μL of the reaction mixture were subjected to electrophoresis with 2% agarose minigel, and the DNA was visualized using UV fluorescence after staining with ethidium bromide. Molecular



(a)



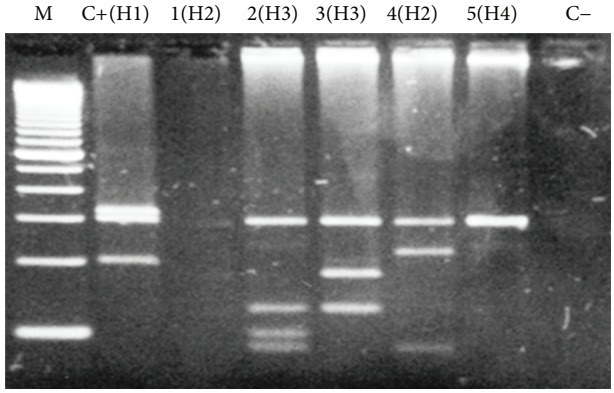
(b)

Figure 1: Automated sequencing (Abi Prism 377) for the ureC region of *H. pylori*. Positive samples for *H. pylori* infection obtained from PCR in order to prove that the sequences being amplified did belong to the genetic sequence of a DNA segment of the urease-C area of *H. pylori (enzymes HhaI and MboI)*. All DNA samples taken from the patients presented genetic sequences similar to the one of *H. pylori*, as described for the urease-C area of the Gene Bank (I Square 1).

weight markers were included in each gel. An 820 base-pair band was seen when samples were amplified using primers P1 and P2 to detect *H. pylori* (Table 1 and Figure 1).

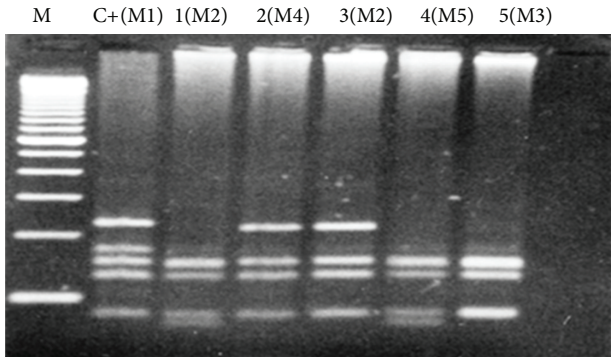
2.2.4. PCR-Based Restriction Fragment Length Polymorphism Typing of Helicobacter pylori (RFLP). After the amplification was confirmed, the PCR product was submitted to digestion with the restriction enzymes HhaI and MboI for fragmentation of Urease-C [16].

The fragments which were produced were submitted to electrophoresis in a 2% gel agarose 1000 (Gibco-BRL), stained with ethidium bromide, visualized under ultraviolet light



M							C–
	241	300	260	260	300	270	
	181	296	240	240	296	267	
	158	114	230	230	114	178	
	140	110	90	90	110	105	

Figure 2: Digestion patterns with the enzyme *HhaI* found in some analyzed samples. Electrophoresis in 2% agarose gel 1000, stained with bromide ethidium. M marker of molecular weight, C+ (1), 1(H2), 2(H3), 3(H4), 4(H4), 5(H2), C-negative control. Note: the most frequent pattern found was H4, with 14 patients (25.8%).



M							C–
	320	308	280	308	300	480	
	300	275	230	275	220	300	
	200	237	120	237	200	40	
			100		100		
			90				

Figure 3: Digestion patterns with the enzyme *MboI* found in some analyzed samples. Electrophoresis in 2% agarose gel 1000, stained with ethidium bromide. M marker of molecular weight; C+(M1); 1(M2); 2(M4); 3(M2); 4(M5); 5(M3); C-negative control. Note 1: Pattern M4 was the most frequent, with 15 patients (25.8%). Note 2: patient 1(M2) presented very clear bands in this analysis, but in a posterior analysis it was possible to classify this patient in group M2.

and photographed in Polaroid System. The patterns which were found were compared and analyzed with the computational program Bio 1D (Analysis of Restriction—PCR-RFLP—Restriction Fragment Length Polymorphism), version 99 (Vilber Loumart) (Figures 2 and 3).

Table 2: Comparison between PCR and urease test in fresh biopsy samples.

	Urease test	PCR
Positive	64	64
Negative	18	18
Total	82	82

100% agreement.

Table 3: Comparison between PCR and histology test in paraffin biopsy samples.

	Histology	PCR
Positive	59	12
Negative	0	47
Total	59	59
Positive *Beta Globin* (DNA detection)	—	14
Negative *Beta Globin* (no DNA detection)	—	45
Total	0	59

* Two paraffin samples were positive for the *Beta Globin* gene, but negative for the *H. pylori* gene.

Approximately 10 μL of the amplified product were used for the digestion process which also contained 2.0 μL of the corresponding enzyme. Water was added to fill 20.0 μL and the mixture was placed in a 37°C bath overnight.

2.2.5. Automatic Sequencing. Automatic sequencing was performed using the program Abi Prism, model 377, version 3.4, and Abi 100, version 3.2. Sequencing allowed for the identification of the studied DNA region (Primers P1 and P2). Figure 1 shows the automatic sequencing, proving that the sequence is *Helicobacter pylori.*

3. Results

A total of 141 endoscopic biopsy samples from 131 patients were studied for *H. pylori* infections with PCR and the results were compared with Urease and Histology tests. 82/64 (78%) fresh samples had a positive Urease test for *H. pylori*. A PCR test detected all of the 64 positive samples identified by the Urease test (100%) (Table 2).

Fifty-nine paraffin biopsies, all found to be positive through a histological examination, were submitted to the DNA extraction procedure and Beta-Globin PCR to prove the quality and the presence of DNA in the extracted samples. Only 14/59 (23.7%) samples were positive for the Beta Globin gene, but in two of them *H. pylori* was not amplified by PCR, even though they had a positive Histology test (Table 3). In the other 45 samples, it was impossible to detect Beta Globin in the DNA using PCR, primarily because of the low amount of paraffin samples and/or because the reaction was inhibited due to paraffin and xilol in the extraction procedures. No contamination occurred and the samples were tested two times.

Among the 141 fresh endoscopic biopsy samples, 58 were tested using the RFLP technique to detect the different *H. pylori* strainswith the restriction enzymes *HhaI* and *MboI*.

Table 4: Use of the Restriction Fragment Length Products (RFLP) technique for genotyping the positive *H. pylori* PCR products using restriction enzymes (*HhaI* and *MboI*).

Restriction enzyme	Frequency (%)	Disease	Median age (years)
HhaI-1	1/58 (1.7)	Gastritis + ulcers	41
HhaI-2	5/58 (8.6)	3 Gastritis; 2 ulcers	29
HhaI-3	**12/58 (20.7)**	**9 Gastritis**; **3 ulcers**;	**45**
HhaI-4	**14/58 (24.1)**	**8 Gastritis**; **3 ulcers**; **1 esophagitis**; **1 Inflamation**	**39**
HhaI-5	3/58 (5.2)	1 Gastritis; 2 ulcers	54
HhaI-6	6/58 (10.3)	3 Gastritis; 2 ulcers; 1 Gastritis + ulcers	39
HhaI-7	1/58 (1.7)	1 ulcers	19
HhaI-8	4/58 (6.9)	1 Gastritis; 2 ulcers; 1 esophagitis	48
HhaI-9	2/58 (3.4)	1 Gastritis; 1 ulcers	37
HhaI-10	2/58 (3.4)	2 ulcers	72
HhaI-11	8/58 (13.8)	6 Gastritis; 1 inflamation; 1 ulcers	54
MboI-1	2/58 (3.4)	2 Gastritis	72
MboI-2	**13/58 (22.4)**	**7 Gastritis**; **5 ulcers**, **1 esophagitis**	**39**
MboI-3	8/58 (13.8)	3 Gastritis; 3 ulcers; 1 inflamation	49
MboI-4	**15/58 (25.8)**	**10 Gastritis**; **5 ulcers**	**57**
MboI-5	4/58 (6.9)	1 Gastritis; 1 ulcers; 1 esophagitis; 1 Gastritis ulcers	27
MboI-6	2/58 (3.4)	1 Gastritis; 1 ulcers	37
MboI-7	4/58 (6.9)	1 Gastritis, 1 splenomegaly; 2 ulcers	50
MboI-8	1/58 (1.7)	1 Gastritis	45
MboI-9	5/58 (8.6)	2 Gastritis; 2 ulcers; 1 Gastritis + ulcers	47
MboI-10	2/58 (3.4)	2 Gastritis	28
MboI-11	1/58 (1.7)	1 Gastritis	31
MboI-12	1/58 (1.7)	1 Gastritis	38

All 58 samples showed positive PCR for Beta Globin and *H. pylori* genes. The product obtained from the *H. pylori* amplification gene by direct PCR was 820 base pairs. Eleven digestion patterns for *HhaI* and twelve for MboI were found (Table 4). The most frequent patterns were HhaI–3 with 12.58 (18.3%), HhaI-4 with 14.58 (23.3%), MboI-2 with 13.58 (21.7%), and MboI-4, with 15.58 (23.3%). The median age was 45, 39, 39, and 57, respectively, in each of the detected patterns. The most frequent diseases in the patients of this study were Gastritis and ulcers.

4. Discussion

Gastric cancer is one of neoplasms that cause the majority of deaths not only in Brazil but all over the world. The type of cancer caused by *H. pylori* could be linked to gastric chronic. Differences in the degree of virulence between strains have lead to an increased risk of developing gastric diseases [17].

The *H. pylori* infection is distributed in a cosmopolitan way, reaching mainly the adult population of low socioeconomic levels in developing countries. The discharge infection rate is correlated with bacterial virulence and inherent factors of the particular host, mainly with respect to the immune system [18].

It should be noticed that the route of fecal-oral transmission appears to be the biggest problem in the prevalence of infection, making *H. pylori* a serious public health problem in both developed and developing countries [19].

The present study analyzes the effectiveness of the molecular biology method PCR in the detection of *H. pylori* in patients with gastrointestinal symptoms, comparing it with the histology and rapid urease test.

The polymerase chain reaction (PCR) for the diagnosis of *H. pylori* is a very sensitive and specific method [20], providing fast and safe diagnosis. Many results indicate that PCR sensitivity is close to that of culture tests [21], but for verifying the eradication of *H. pylori* the effectiveness of PCR can be markedly superior [22, 23]. The methodology used in other studies to distinguish the different *H. pylori* subtypes has been PCR-RFLP [24] that through analyzis of the PCR product with restriction endonucleases that resulting fragments of different sizes and the digestion profile is decisive to define the strains. The restriction enzymes HhaI and MboI were used for the Urease-C area [16]. The extreme degree of variability observed among the strains of *H. pylori* became an important focus of scientific attention, as the investigators recognized the significant impact that this phenomenon can have on several research areas, such as the development of vaccines, the development of resistance to antimicrobial agents, and the study of the pathogen-host interaction [25, 26]. Considering that 10 to 20% of people infected with *H. pylori* develop obvious diseases, the reliable identification of the lineages could actually be very beneficial [27]. Previous studies that

have used several techniques characterized *H. pylori* as a highly variable species that presents countless lineages, each one with its own and different genotype [28–31].

The genotyping of *H. pylori* is important for characterizing the most pathogenic genotype and the most frequent strain. This information can be used for clinical and epidemic studies. Even if many infections are clinically silent, the organism infected with *H. pylori* presents increased morbidity and mortality [5, 32, 33].

In the present study we standardized PCR with material obtained from the fresh endoscopic biopsies samples of patients attending Gastrocentro (the Center of Digestive Tract Studies), Medical School, UNICAMP. Some of the gastric biopsy samples were collected in paraffin and some were not. With regard to the standardization of the DNA extraction technique from the paraffin biopsy samples, several difficulties were found, because the samples contained a small amount of tissue fragments and many of the paraffin samples did not amplify the β-Globin gene, demonstrating degraded DNA of poor or inhibited quality.

PCR was used because it is more specific and faster when compared to other methods; the product of PCR can be processed with restriction enzymes to verify *H. Pylori* strains. Besides, starting with the PCR, DNA sequencing can be made to verify mutations, which no other technique is capable of doing.

As an internal control of the reaction was used in all samples (human β-Globin gene), in the fresh-air biopsy samples positive for *H. pylori*, we had 100% PCR amplification. However, in several paraffin samples, the β-Globin did not amplify, indicating an inefficient DNA extraction of the samples.

The efficiency of *H. pylori* detection PCR in fresh samples was superior to that in the paraffin samples. We suspect that PCR inhibition may have occurred due to the method used in DNA extraction from paraffin or the fact that that the samples were insufficient.

The extraction of DNA from fresh samples had excellent results. Among the 82 analyzed samples, 64 were positive and 18 negative, with 100% in agreement with PCR.

In the present study, we used the PCR-RFLP method for the differentiation of *H. pylori* strains from specimens obtained from gastric biopsies taken from Brazilian patients. Using this methodology we observed that 12 and 11 patterns were produced, respectively, by the two restriction enzymes *MboI* and *HhaI* from 58 specimens obtained from gastric biopsies. Two were samples of biopsies in paraffin and 58 were samples of nonfastened gastric biopsies (Table 1).

This data suggests that genotyping using PCR-RFLP can be useful as a fast procedure for the specific identification of *H. pylori* lineages in gastric biopsies specimens [16]. Several protocols of genotyping analysis were proposed for distinguishing the lineages of clinically isolated *H. pylori* [34–38]. Several primer pairs were described for detection and the typing of *H. pylori* was based on the amplification of the ureA [34], ureA plus ureB [35], and ureC genes [36, 38]. These results demonstrate great diversity in the urease genes in clinical *H. pylori* samples. Li et al. [16] found 3, 11, and 6 different patterns which were produced by 19 clinically isolated samples, respectively, digested by the restriction enzymes *HhaI, MboI* and AluI. Foxall et al. [35] found 10 different patterns which were produced by 22 clinically isolated samples, when the restriction enzyme *HaeIII* digested the PCR product of 2.4 Kb which had been amplified by the ureA and ureB genes. Lopez et al. [37] found that the patterns generated by the digestion of PCR products with the *HaeIII* enzyme, starting from ureA and ureB, were almost as different as the standard *HaeIII*. Akopyanz et al. [28] found 18 *MboI* and 27 *HaeIII* RFLP patterns, PCR products amplified by ureA and ureB genes of 2.4 Kb of 60 *H. pylori* lineages, and that the patterns distinguished 44 separate groups. Each isolated group did not differ from the other ones in the RFLP analyses of ureA and ureB products, but differed in *MboI* digestion of the 1.7 Kb ureC and ureD segments. Such a fact indicates that PCR-RFLP analyses of ureC genes can produce a great number of standard RFLPs.

Several studies have confirmed that PCR-RFLP analysis of the ureC gene can differentiate clinically isolated *H. pylori*. Using restriction endonucleases, Moore et al. [38] analyzed the 1.1 Kb portion of the ureC gene amplified by the "PCR" of 21 clinically isolated *H. pylori*. The samples were divided into four groups after digestion with the enzyme *HindIII*, while the lineages were divided into 15 groups after they were digested with the enzymes *AluI* and *PvuI*. Fujimoto et al. [36] demonstrated that the digestion of 820 bp of the *H. pylori* ureC gene with the restriction enzymes *HhaI*, *MboI,* and *MseI* resulted in 10, 10, and 11 different patterns, respectively. Dooley et al. [3] used three types of enzymes in the PCR product of a 1.179 bp portion of the *H. pylori* ureC gene. Eleven, 10, and 6 digestion patterns were produced by the *HhaI, MboI,* and *AluI* enzymes, respectively.

In our study we used two types of restriction enzymes in the amplified products of 820 pb of the *H. pylori* ureC gene. We obtained 11 and 12 different patterns, respectively, from the 58 clinically isolated samples which were studied. Our results suggest that the PCR-RFLP analysis of this portion of the *H. pylori* ureC gene is a reliable method, and that genotyping of PCR in this area can be used for epidemic studies and for the differentiation of isolated *H. pylori strands*.

This study also revealed a high level of genetic diversity isolated in the different *H. pylori* positive patients studied in Brazil.

The obtained genotyping patterns were compared using the computational program Bio 1D. We found 11 patterns with the *HhaI* enzyme and 12 patterns with the *MboI* enzyme. The reason for the small number of studied samples was due to the fact that it was not possible to establish a significant statistical correlation between specific digestive pathologies and standard genotypes.

We believe that the genotyping of *H. pylori* can contribute to the study of the microorganism's characteristics, facilitating the detection of pathogenic or nonpathogenic strains and, in turn, providing a better understanding of the several virulence factors that the bacterium uses to cause diseases.

4.1. Statistics. Percentage agreement was calculated to compare *H. pylori* genotypes obtained from PCR performed directly on gastric biopsies, with the genotypes obtained from

the PCR of DNA extracted from paraffin and fresh samples, as well as histology and urease tests.

Conflict of Interests

Authors have no conflict to declare.

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Clinical Study

Comparing Multiplex PCR and Rapid Urease Test in the Detection of *H. pylori* in Patients on Proton Pump Inhibitors

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Background. This study was conducted to assess the diagnostic value of a multiplex PCR assay to detect *H. pylori* infection and to further evaluate the negative results from the CLOtest on patients with and without PPI treatment. Methods. This study is a retrospective cohort that included 457 patients with symptoms of dyspepsia, who underwent upper endoscopy at Evanston and Glenbrook Northshore Hospital from June 2003 to October 2007. A total of 556 samples were reported with some patients having more than one test over the time period. The CLOtest was performed first on the gastric specimen and from that specimen, the DNA was isolated and the one-step multiplex PCR was performed. *Results*. By M-PCR testing, *H. pylori* was detected in 143 (52.2%) of 274 cases in the control group and 130 (46.1%) of 282 cases in patients on PPI treatment ($P = 0.1746$). The CLOtest detected the presence of *H. pylori* in 4 (1.4%) of 282 cases from the same group receiving PPI treatment and 29 (10.6%) of 274 cases from the group not taking a PPI ($P \leq 0.0001$). *Conclusion*. Our PCR is sensitive enough to detect the presence of *H. pylori* despite being on PPI treatment.

1. Purpose

Helicobacter pylori (*H. pylori*) is a spiral-shaped bacterium that is found primarily in the stomach [1]. The bacteria has a significant pathogenic role in gastritis, gastric cancer, gastric mucosa-associated lymphoid tissue lymphoma, and peptic ulcer disease [2]. The World Health Organization classifies *H. pylori* as a class I carcinogen [3]; this is a major concern because approximately half of the world's population are infected with *H. pylori* [4].

Currently, there are numerous tests available to identify *H. pylori*, but there is no gold standard. Rapid urease test (RUT) is widely used in clinical practice to detect the urease enzyme of *H. pylori* in gastric mucosal biopsies. The urease enzyme hydrolyzes urea into carbon dioxide and ammonia allowing *H. pylori* to survive in the acidic medium [2, 5]. It is commonly believed that acid-reducing drugs, in particular proton pump inhibitors (PPIs), decrease the sensitivity and accuracy of the RUT, the urea breath test, histology, and the stool antigen test by reducing the amount of *H. pylori* [6, 7].

Proton pump inhibitors decrease the activity of *H. pylori* within the stomach and shift their distribution proximally. It is proposed that PPIs inhibit the growth of *H. pylori* through a pH-dependent mechanism. Proton pump inhibitors can cause false negatives in diagnostic tests and should be stopped for at least 2–4 weeks before performing a test [8, 9]. However, this generates a problem because PPI withdrawal is strongly associated with symptom recurrence. While on a PPI, a negative RUT is insufficient to rule out an infection. The biopsy specimen may contain low bacterial density of viable cells, giving a negative result. This becomes an issue as many Americans are taking these medications. In 2009, PPIs ranked third in US sales and sixth in the total numbers of prescriptions dispensed [10]. In several studies, the authors concluded that PPIs reduce the sensitivity and specificity of the antral and corpus biopsies for RUT and histological

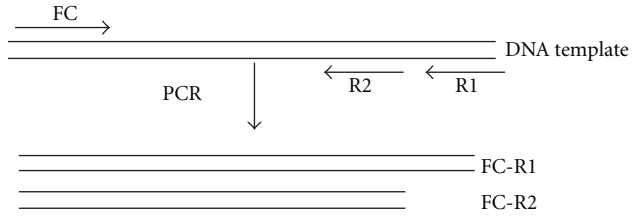

Figure 1: Diagram of primers designed for each locus. FC, the forward primer, is the common primer; R1 and R2 are the pair of reverse primers. The amplicons FCR1 and FC-R2 are amplified from each locus.

examination. The polymerase chain reaction (PCR) is more sensitive in detecting *H. pylori*. Yakoob et al. showed that the PCR is more sensitive than RUT and histology in patients taking PPIs [7, 11, 12]. However, the problem of single-gene PCR still has less than ideal specificity and false positives. With the problems that acid-reducing drugs cause to many diagnostic tests of *H. pylori*, the mutation rates of DNA, and current PCR methods testing for 1 or 2 genes, we developed a unique multiplex PCR (M-PCR) that detects 5 unique genes, improving the specificity.

In a previous study we conducted, our unique M-PCR accurately identifies *H. pylori* compared to RUT and immunohistochemical analyses; in addition to identifying significant number of *H. pylori* infections that would not be detected by the former methods [13–15]. The aim of this study is to determine the effect of PPIs on the results of the RUT and M-PCR. We hypothesize that M-PCR will not be affected by the physiological changes from PPIs due to the sensitivity of M-PCR technology and stability of DNA.

2. Methods

2.1. Patients. This study is a retrospective cohort that included 457 patients with symptoms of dyspepsia, who underwent upper endoscopy at Evanston and Glenbrook Northwestern Hospital from June 2003 to October 2007. Biopsies were taken at the gastric antrum and body. The study was divided into two groups based on a comprehensive chart review: the first group was on a PPI and the control group was not on a PPI for at least four weeks. Those taking H_2-receptor antagonists and antibiotics within the past 4 weeks before the endoscopy were excluded from both groups. Informed consent was obtained from each patient, and the study was reviewed and approved by the Evanston Northwestern Health Care Institutional Review Board.

2.2. Rapid Urease Test (CLOtest). The CLOtest rapid urease test (Kimberly-Clark, Roswell, GA, USA) was performed first on all the gastric specimens according to the manufacturer's instructions. A definite magenta color was required to read the test as positive. The results were interpreted after 20 minutes and then 24 hours later.

2.3. Multiplex PCR. After the CLOtest was read, the same specimen was sent to the laboratory to isolate the DNA.

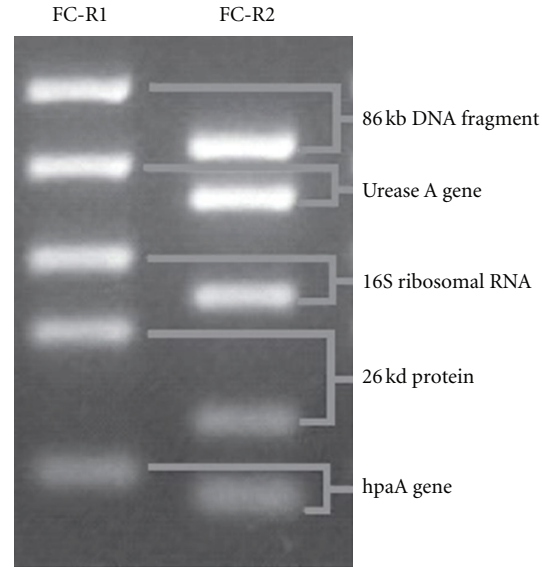

Figure 2: Five pairs of DNA bands amplified from the 5 targeted loci specific for *Helicobacter pylori*.

Then the one-step M-PCR was performed. The researcher evaluating the M-PCR electrophoresis gel was blinded to the CLOtest results. The M-PCR targeted the following loci: 0.86-kb DNA fragment, urease A gene, 16S ribosomal RNA, 26-kDa protein antigen, and hpaA gene. For each locus, one forward primer, the common primer (FC), and two reverse primers (R1 and R2) were selected. The R2 primer is located inside the amplifying region of R1. The R1 and R2 primers were mixed with five FC primers, respectively, and set in two separate amplification systems of FC-R1 and FC-R2 primers (Figure 1). A total of 10 DNA fragments could be amplified, in 2 tubes, each containing 5 amplicons internal to the other. For the M-PCR, we define a positive case for *H. pylori* if 5 of the 10 fragments or two sets of DNA fragments from the same locus were amplified because of the high diversity of DNA sequences of the bacteria (Figure 2) [13–16]. In each M-PCR run, positive (strain J99) and negative (water blank) control samples are assayed to ensure that there is a reference and no contamination. Each M-PCR performed contains a negative control that contains all reaction components except gastric tissue to assess for contamination. Also, three physically separate work places were set up for template preparation, PCR reactions, and post-PCR analysis to avoid contamination. Special aerosol-resistant pipette tips and routine UV and alcohol cleaning were used.

2.4. Statistical Analysis. The statistical package used was Graph InStat Version 3.10. Statistical analysis was performed by using the Fisher Exact test, 2-tailed. P values of less than 0.05 were considered significant.

3. Results

A total of 556 samples were reported with some patients having more than one test over the time span in which data

Table 1: Difference between M-PCR and CLOtest.

	On PPI $n = 282$	Without PPI $n = 274$	P value
CLOtest			
Positive	4 (1.4)	29 (10.6)	<0.0001
Negative	278 (98.6)	245 (89.4)	
M-PCR			
Positive	130 (46.1)	143 (52.2)	0.1746
Negative	152 (53.9)	131 (47.8)	

was collected. A postclinical record review indicated that there were 282 (50.7%) cases where people were taking a PPI before endoscopy for at least four weeks.

There was no difference between the two groups tested by M-PCR. By M-PCR testing, *H. pylori* was detected in 143 (52.2%) of 274 cases in the control group and 130 (46.1%) of 282 cases in patients on PPI treatment ($P = 0.1746$). The CLOtest detected the presence of *H. pylori* in 4 (1.4%) of 282 cases from the same group receiving PPI treatment and 29 (10.6%) of 274 cases from the group not taking a PPI ($P \leq 0.0001$). The M-PCR identified *H. pylori* in 33 (97.1%) of the 34 cases from the CLOtest (Table 1).

In both the PPI and no PPI groups, there was a significant difference in detection rates between the CLOtest and M-PCR ($P \leq 0.0001$). Additional 241 (46.1%) of 523 cases were detected by M-PCR that were CLOtest negative. Specifically in the PPI group, 127 additional cases out of 278 (45.7%) were detected and 114 out of 245 (46.5%) in the control group were detected.

4. Conclusion

H. pylori is known to be a major human pathogen. Because of the diverse effects of *H. pylori*, an accurate detection method is needed. Currently, there is no one method that is sufficiently sensitive and specific to be considered "gold standard," so we could not use a standard to compare; but used what is commonly used in practice with proven clinical significance. We developed a unique multiplex PCR assay to detect *H. pylori* in endoscopic biopsy specimens.

This study has demonstrated that PPIs affect the *H. pylori* detection rate by CLOtest method, but not the M-PCR. This is an important factor to consider when choosing a diagnostic test to detect *H. pylori*. Also, the results showed additional 46.1% positive cases by retesting the negative results by the CLOtest. Therefore, *H. pylori* testing by current methods should be carefully reviewed, especially the patients who have recently been taking PPIs to ensure that the result is not a false-negative. The CLOtest is highly specific but requires a high density of bacteria for detection. The M-PCR is sensitive enough to detect the presence of *H. pylori* despite an individual being on PPI treatment.

The high detection rate of the M-PCR in our study can be attributed to our study patient population. We only tested patients that were symptomatic, which are more likely to have an infection; as a result the numbers would be higher in these types of patients. Another source for the difference in detection rates and low detection rates in the RUT between the groups is from the formation of the coccoid forms of *H. pylori*. It can exist in three stages: spiral, viable coccoid, and degenerative unviable coccoid form. The coccoid forms can be induced by various conditions, such as PPI and certain antibiotics [17]. Studies have shown that the protein content and genetic material remain unchanged during the conversion from spiral to coccoid forms. The urease activities of the coccoid cells are lower than the spiral form [18]. Identification of the coccoid forms by RUT is difficult; however, PCR methods are used to detect the genetic material since DNA stays intact. A study by Can et al. showed the reliability of the ureA gene region in the coccoid form, which was induced by different factors since no mutations were detected [19]. We have the assumption shown by previous studies that our M-PCR is able to detect the DNA in the coccoid form. The coccoid form may be less virulent and less likely to colonize and induce inflammation. However, it may play a role in infection and is suspected to be partly responsible for relapse of infection after antimicrobial treatment. When the conditions become suitable, the coccoid form can revert back to the spiral form and may regain infectivity [18].

We did not exclude other medications other than the ones mentioned. Any drug that increases pH or an antibiotic can affect the growth of *H. pylori* thus possibly affecting the results. One patient tested positive by the CLOtest, while negative by the M-PCR. There could be several reasons for this discrepancy; it could be a false positive where the CLOtest is 97% specific or where a different urease producing enzyme bacteria or the M-PCR could not detect the bacteria due to a gene mutation. In our previous study, all the positive patients by immunohistochemical analysis and the CLOtest were also positive through the M-PCR method in gastric specimens [13].

Our positive M-PCR results of 46.1% and 52.2%, PPI and control groups, respectively, are similar to our previous study that detected 52% of the cases with *H. pylori* and an additional 40% from the negative results. We conducted a blinded study that correlated the detection rates of the M-PCR to inflammation scores, immunohistochemical findings, and CLOtest results. The M-PCR and CLOtest results were not known by an independent pathologist who examined the histological features. The study concluded that in gastric biopsy specimens the average activity and chronic inflammatory scores were significantly greater in PCR-positive than in PCR-negative, showing the presence of *H. pylori*. In the gastric biopsy specimens, the M-PCR detected *H. pylori* in all the positive cases detected by immunohistochemical analysis and/or CLOtest [13].

The results of the study and findings are consistent with those of a previous study conducted by Yakoob et al. The study found no difference in the detection rates by a PCR between the group that was on a PPI and the control group, 74% versus 75% in the antrum. It also concluded that the diagnostic yield of both RUT and histology was reduced and PCR is more sensitive than both. In the PPI group biopsied from the antrum, 74% in the PCR, 18% in the

RUT, and 50% in the histology were tested positive. Also, additional 68% in the PPI group and 44% in control were found to be positive by PCR. The detection rates for the PCR are higher compared to our study and this can be due to the smaller study population, ethnic background, or sicker patients. Also, the detection rate may be higher due to the fact that their study used one gene versus our studying using 5 genes. The study concluded that the use of acid reducing drugs decreased the diagnostic yield of RUT compared to the PCR and histology [11]. In most other studies involving traditional PCR, one or two genes were used to identify *H. pylori*. Our M-PCR differs from all other studies since our M-PCR uses 5 genes, and therefore, is more specific for *H. pylori*. So it may be difficult to correlate the results of our M-PCR to traditional PCR.

The use of PCR technology for detection of microorganisms, including *H. pylori*, is well documented. Potential problems with traditional PCR methods include false positives due to contamination and homological DNA sequences among various species. In our study, there was a large difference between the M-PCR and CLOtest in the non-PPI group that may represent false positives [20]. The primers used for the M-PCR were scanned across the gene bank of the National Center for Biotechnology Information and no matches were found. Also the M-PCR for *H. pylori* was tested against 19 bacterial species (*E. coli, Enterobacter aerogenes, Enterobacter cloacae, Enterococcus* species, *Viridians* Group, *P. aeruginosa, Serratia* species, *Klebsiella pneumoniae, MRSA, Lactobacillus* species, *Citrobacter* species, *Bacteroides fragilis (ATCC25285), Wolinella succinogenes (ATCC29543), Campylobacter Jejuni (ATCC33291), Helicobacter pullorum (ATCC52802), Helicobacter fennelliae (ATCC35683), Helicobacter* species *(ATCC35683), Helicobacter heilmannii (ATCC49286), and Helicobacter felis (ATCC49179)*). None of the 19 bacteria showed the standard *H. pylori* M-PCR band patterns. There is also an internal control to prevent false positives. Our M-PCR amplifies 10 DNA fragments at the same time as well as two fragments that will be produced for each of the five loci, one internal to the other. The internal control of our one-step multiple-nested PCR is used to rule out false-positives caused by homological DNA sequences among various species in the primer binding sites, making the M-PCR more specific than the traditional PCR. Also as previously stated, our M-PCR test was validated through our study that showed that our positive M-PCR results showed significantly greater average activity and chronic inflammatory scores.

The detection rates of RUT in non-PPI group were low in the study. This may have been attributed to other acid-reducing medications we did not exclude like bismuth compounds or calcium products. Although we went through a comprehensive medication review and survey, patients may not have disclosed full information. Our detection rates were lower than other studies, but other studies mentioned have yielded low rates as well [11, 12].

Overall, the M-PCR detected an additional 241 positive cases. If a patient has a negative result from a RUT, our M-PCR has proven useful in the diagnostics of *H. pylori* to further evaluate the negative result, as it is not affected by acid reducing drugs. The use of M-PCR can be recommended as an additive test to confirm the presence of *H. pylori* in patients with a negative RUT. Also, for clinicians who require their patients to be on empirical treatment or maintenance therapy, the M-PCR assay can be used so the patient does not need to be taken off a PPI. This M-PCR assay identified a significant number of *H. pylori* infections that would not be detected by RUT, finding additional 46.1% positive cases. Our M-PCR for *H. pylori* will increase detection rates, increasing opportunities for medical interventions and allowing for patients to be treated through sensitive and specific method that is not affected by PPI unlike many other diagnostic methods. We developed an available M-PCR in the United States available for physicians to utilize.

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Research Article

Reduced FAF1 Expression and *Helicobacter* Infection: Correlations with Clinicopathological Features in Gastric Cancer

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Background. This study aimed to investigate possible associations between FAF1 expression and aspects of gastric cancer, in particular its clinical characteristics and *Helicobacter* infection. Materials and Methods. RT-PCR and immunohistochemistry were used to analyze expression of FAF1 mRNA and protein in 40 gastric cancer patients.*H. pylori* infection was detected by three staining protocols. Results. The expression level of FAF1 mRNA was significantly lower in gastric cancer tissue than in normal gastric mucosa from the same patient ($P < 0.05$). FAF1 mRNA expression was significantly lower in stage IV gastric cancer than in stage I+II or IIIA+IIIB ($P = 0.004$) and also significantly lower in gastric cancer with distant metastasis. FAF1 mRNA expression was higher in well-differentiated cancer than in poorly-differentiated cancer (0.39 ± 0.06 versus 0.19 ± 0.06, $t = 9.966$, $P < 0.01$). FAF1 protein was detected in 15 of 40 (37.5%) cancerous tissue samples and in 29 of 40 (72.5%) corresponding normal tissue samples ($P < 0.01$). FAF1 mRNA expression was lower in *H. pylori*-positive cancerous tissue samples than in *H. pylori*-negative ones ($P < 0.05$). Conclusions. Downregulation of FAF1 expression may be related to the carcinogenesis and progression of gastric cancer, and *H. pylori* infection during gastric carcinogenesis may downregulate FAF1 expression.

1. Introduction

Recently Fas-associated factor 1 (FAF1) was discovered and characterized as a potent regulator of cell survival, facilitating apoptosis by interacting with Fas-associated death domain (FADD), caspase-8, and protein kinase CK2-β [1–3]. It is a component of the death-inducing signaling complex, and when overexpressed, it can initiate apoptosis and induce cell death in some cell types even in the absence of any extrinsic death signals [1]. FAF1 binds death effector domain-interacting domain (DEDID), and it interacts with the death domain of Fas through its Fas-interacting domain (FID) [4, 5].

Subsequent work has revealed that the FID of FAF1 interacts with a variety of downstream targets, and functional loss of FAF1 may provide a prosurvival signal to cells in disease states such as cancer [6]. In fact, loss or downregulation of FAF1 expression has been observed in various human cancers [7, 8]. Western blotting studies have shown that FAF1 protein levels are lower in human gastric carcinoma tissue than in healthy control tissue from the same patients [9]. However, it remains unclear whether the lower protein levels reflect lower levels of FAF1 gene expression, and whether that downregulation is associated with the clinical characteristics of gastric cancer, including the often-observed comorbidity of gastric infection by *Helicobacter pylori*.

Gastric carcinoma is a major cause of morbidity and mortality worldwide. The precise mechanism of gastric carcinogenesis is not yet fully understood. *H. pylori* is a Gram-negative bacillus capable of colonizing the gastric mucosa. Countries with high rates of gastric cancer, such as China and Japan, tend to have a high prevalence of *H. pylori* infection [10]. Although *H. pylori* infection is considered one of the earliest steps in gastric carcinogenesis, how it contributes to the disease remains obscure [11, 12].

In the stomach, a homeostatic balance is maintained between the proliferation and apoptosis of gastric mucosal cells, and changes in this balance seem to be the biological

basis of gastric carcinogenesis [13]. There is increasing evidence that apoptosis plays an important role in the pathogenesis of a variety of diseases caused by bacteria, viruses, and other factors; apoptosis is also regarded as one of the most important mechanisms of tumor cell suicide [14, 15]. The process of apoptosis involves a wide variety of regulatory and effector molecules, and Fas is one of the most important groups of apoptosis regulators [16].

Increasing evidence indicates that bacterial pathogens modulate the apoptotic signaling cascade of host cells and thereby cause disease [15]. However, we are unaware of studies examining whether *H. pylori* infection is associated with changes in FAF1 expression during gastric carcinogenesis. In the present study, we investigated this and other possible associations between clinical characteristics of gastric cancer and FAF1 expression.

2. Methods

2.1. Patients and Design. This study involved 40 patients with gastric cancer (29 male, 11 female) who were referred for surgery between June 2005 and April 2006. Patients had either been admitted directly to the Affiliated Tumor Hospital of Guangxi Medical University, or they had been admitted first to the People's Hospital of Guangxi Zhuang Autonomous Region and then referred to the Affiliated Tumor Hospital. Patient age ranged from 34 to 78 years (median 55). None received neoadjuvant treatments. Prior informed consent was obtained, and the study protocol was approved by the Ethics Committees of both the Affiliated Tumor Hospital and the People's Hospital.

Tissue specimens from both the cancerous lesion and from normal gastric mucosa located more than 5 cm from the primary tumor were collected from each patient at the time of surgery. Specimens were immediately frozen in liquid nitrogen, and stored at −80°C until use. All cases were reviewed by two specialists to confirm diagnosis according to the criteria of the Japanese Gastric Cancer Association [17].

Specimens of paracancerous tissue, defined as noncancerous gastric tissue located less than 2 cm from the primary tumor, were collected at the time of surgery, fixed in 10% neutral formalin, and embedded in paraffin. Serial thin sections were prepared and placed on glass slides coated with 3-aminopropyltriethoxysilane (Sigma, USA), then stained as described below to detect FAF1 protein and *H. pylori* infection.

2.2. cDNA Synthesis and RT-PCR. Gastric tissue specimens (approximately 0.05–0.1 g) were homogenized with a homogenizer, and total RNA was extracted with a Trizol kit (Invitrogen, USA). Total RNA (3 μg) from each sample was reverse-transcribed into cDNA using the AMV Reverse Transcription system (Promega, USA). As a reference for normalizing levels of FAF1 mRNA, part of the β-actin housekeeping gene was amplified using 2 μL of cDNA from each sample.

Primers were designed using Primer Premier 5.0 (Premier Biosoft International, USA) based on published mRNA sequences of FAF1 (GenBank NM_007051.2) and β-actin (NM_001101). The sequences of the PCR primers were: FAF1, 5′-cttgctgaatcagggctctc-3′ (Forward) and 5′-tccaccccaaattctgtagc-3′ (Reverse) to give a 164-bp product; and β-actin, 5′-accgagcgcggctacagc-3′ (Forward) and 5′-ctcattgccaatggtgat-3′ (Reverse) to give a 180-bp product.

RT-PCR was performed using a SYBR Green kit (Applied Biosystems, USA) in an ABI Prism 7300 HT Sequencer (PE Applied Biosystems, USA) according to the manufacturer's instructions. The following cycling parameters were used: 5 min at 94°C; 35 cycles of 45 s at 94°C, 45 s at 55°C and 1 min at 72°C; and finally 10 min at 72°C. Levels of FAF1 mRNA were calculated based on the threshold cycle (CT) values and normalized to levels of β-actin mRNA. The results were analyzed using the $2^{-\Delta C_T}$ method and the formula $\Delta C_T = \text{Avg} \cdot \text{FAF1 } C_T - \text{Avg} \cdot \beta\text{-actin } C_T$.

2.3. Immunohistochemistry. FAF1 protein was detected by immunohistochemistry using a monoclonal anti-FAF1 antibody (clone 1A10, Santa Cruz Biotechnology, USA) at a 1 : 100 dilution. Antibody staining was carried out strictly according to the manufacturer's instructions. Paraffin sections of rat kidney served as a positive control. As negative controls, experimental patient specimen slides were prepared and subjected to the normal antibody staining procedure but with the primary antibodies omitted. Immunolabelling was detected using an avidin-biotin complex and 3,3′-diaminobenzidine as chromogen (Fuzhou Maixin Biotechnology Development Co. Ltd., China). Sections were counterstained with hematoxylin.

2.4. Evaluation and Scoring of FAF1-Positive Cells. For each patient, four peripheral fields and a middle field were selected, and the numbers of cells positive for FAF1 protein were determined for a sample of 500 tumor cells and for 500 corresponding normal gastric mucosa cells. Slides were semiquantitatively evaluated by two pathologists working independently (Y. Luo and X.-q. Ye). If the percentage of cells positive for FAF1 protein was <6% of the 500 cells examined, then the sample was judged to be negative for FAF1 protein expression; if the percentage was ≥6%, it was considered positive. Average values were determined for all cancerous tissue samples and noncancerous tissue samples.

2.5. Detection of H. Pylori Infection. Samples of normal gastric tissue, paracancerous tissue, and primary tumor tissue were taken from each patient. To reduce the risk of false positives, the method of Madan et al. [18] was used. Briefly, thin sections (4 μm) from each tissue sample were stained with hematoxylin and eosin (H&E), toluidine blue, and Warthin-Starry silver in order to detect *H. pylori* infection. Samples had to be positive by at least two staining methods in order to be judged *H. pylori*-positive. Samples were divided into a subgroup of *H. pylori*-negative cancerous samples and a subgroup of *H. pylori*-positive cancerous samples.

2.6. Statistical Analysis. A database of clinicopathological information on the 40 patients was set up in SPSS 16.0

(SPSS, Inc., USA), which was used to analyze the data for associations and conduct all statistical tests. Measurements are presented as the mean ± SD. Differences in FAF1 mRNA expression between gastric cancer tissue samples and the corresponding normal gastric mucosa samples were examined using the paired t-test and a test for homogeneity of variance. Differences in FAF1 mRNA expression between the cancerous and normal groups on one hand and clinicopathological features on the other were examined using the independent-samples t test and one-way ANOVA. Differences in percentages of cells positive for FAF1 protein were examined using χ^2 analysis. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Expression of FAF1 mRNA and Protein in Gastric Cancer. Expression of FAF1 mRNA and β-actin mRNA was detected in all 40 gastric cancer samples and corresponding normal gastric mucosa samples. FAF1 mRNA expression was significantly lower in gastric cancer samples than in the matched normal gastric mucosa samples (0.27±0.12 versus 0.48±0.08, $t = 9.209$, $P < 0.05$) (Figure 1). Consistent with this finding, FAF1 protein was detected in only 15 of 40 (37.5%) gastric cancer samples, compared to 29 of 40 (72.5%) corresponding normal gastric mucosa samples ($\chi^2 = 9.899$, $P < 0.01$). FAF1 protein staining localized primarily to the nucleus and in some cases to the cell membrane (Figure 2).

We performed various subgroup analyses to examine possible associations between FAF1 mRNA expression and clinicopathological features of gastric cancer. We examined patient gender, age, size, histological grade, invasion depth, lymph node metastasis, distant metastasis, and clinical staging. FAF1 mRNA expression level was lower in stage IV gastric cancer tissue (0.18 ± 0.12) than in Stage I + II (0.32 ± 0.12) or stage IIIA + IIIB cancer tissue (0.30±0.11, $F = 6.276$, $P = 0.004$), and it was lower in gastric cancer with distant metastasis than in gastric cancer without distant metastasis (0.19 ± 0.07 versus 0.29 ± 0.12, $t = -2.753$, $P < 0.01$). Conversely, the FAF1 mRNA expression level was higher in well-differentiated cancer tissue than in poorly differentiated cancer tissue (0.39 ± 0.06 versus 0.19 ± 0.06, $t = 9.966$, $P < 0.001$). FAF1 mRNA expression showed no obvious association with gender, age, tumor size, infiltration degree, lymph node metastasis or clinical stage below IV in gastric cancer (Table 1).

Table 1: FAF1 mRNA expression and clinicopathological characteristics of gastric cancer.

Parameter	n	FAF1 mRNA level**	$t(F)$	P
Gender				
Female	11	0.27 ± 0.12	0.192	0.849
Male	29	0.26 ± 0.11		
Age (years)				
≥60	17	0.27 ± 0.12	0.53	0.599
<60	23	0.25 ± 0.11		
Tumor size (cm)*				
≥5	18	0.26 ± 0.11	0.1	0.921
<5	22	0.26 ± 0.12		
Histological grade				
Well differentiated	14	0.39 ± 0.06	9.966	<0.001
Poorly differentiated	26	0.19 ± 0.06		
Invasion depth				
No serosa invasion	7	0.30 ± 0.12	1.09	0.283
Serosa invasion	33	0.25 ± 0.11		
Lymph node metastasis				
Present	3	0.34 ± 0.14	1.266	0.213
Absent	37	0.25 ± 0.11		
Distant metastasis				
Present	12	0.19 ± 0.07	−2.753	0.009[a]
Absent	28	0.29 ± 0.12		
Clinical stage				
I + II	8	0.32 ± 0.12	6.276	0.004[a]
$III_A + III_B$	17	0.30 ± 0.11		
IV	15	0.18 ± 0.12		

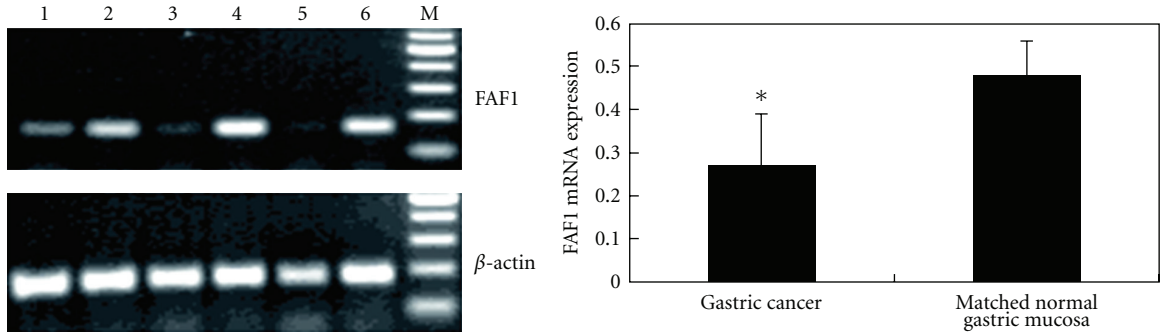
*Defined by the longest dimension in the gastric mucosa.
**Normalized to the level of β-actin.
[a]$P < 0.01$, based on a comparison using a mean ± SD test.

3.2. Prevalence of H. Pylori Infection. *H. pylori* was detected by three staining methods: it appeared light purple by H&E, brownish-black by Warthin-Starry silver and light blue by toluidine blue (Figure 3). *H. pylori* was found to exist primarily in the paracancerous tissue; it was rarely found in necrotic cancer tissue, perhaps because the microenvironment was unsuitable for its growth. It was usually found clustered in glandular organ cryptae of gastric mucosa. Based on H&E staining, 25 of 40 (62.5%) biopsies were positive for *H. pylori,* including 6 biopsies negative by Warthin-Starry silver staining and 5 negative by toluidine blue. Only 1 biopsy was positive by Warthin-Starry silver and toluidine blue, but negative by H&E. Applying the criterion that biopsies must be positive by at least two staining methods to be considered positive for *H. pylori,* our results indicate an infection rate of 21 of 40 (52.5%).

3.3. Association between FAF1 mRNA Expression and H. Pylori Infection. FAF1 mRNA expression was lower in *H. pylori*-positive tissue samples than in *H. pylori*-negative samples from gastric cancer patients (0.18 ± 0.06 versus 0.29 ± 0.12, $t = 3.6084$, $P < 0.05$). In contrast, among normal gastric mucosa samples from the same patients, FAF1 mRNA expression did not vary with the presence or absence of *H. pylori* infection (0.49 ± 0.08 versus 0.47 ± 0.11, $t = 0.6515$, $P > 0.05$).

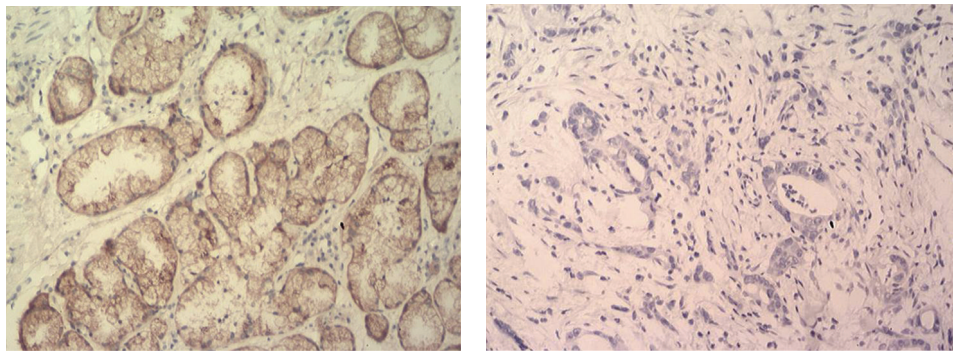
4. Discussion

The recurring loss or downregulation of FAF1 expression observed in certain human and murine cancers suggests that this proapoptotic factor is a tumor suppressor, though FAF1 expression has yet to be analyzed in detail in several other human cancers, including gastric cancer [19, 20].



(a) (b)

Figure 1: RT-PCR analysis of FAF1 mRNA expression in gastric cancer tissue and matched normal gastric mucosa tissue. (a) RT-PCR to measure levels of FAF1 and β-actin mRNA. Lanes 1, 3, 5: gastric cancer tissue from different patients. Lanes 2, 4, 6: corresponding normal gastric mucosa tissue from the same patients. M, 100-bp DNA marker ladder. β-actin was used as an internal control. (b) Comparison of FAF1 mRNA levels (normalized to β-actin levels) in gastric cancer tissue and matched normal gastric mucosa tissue. FAF1 mRNA levels were much lower in the gastric cancer tissue samples based on the mean ± SD test. $^*P < 0.05$.



(a) (b)

Figure 2: Immunohistochemistry to detect FAF1 protein. (a) Normal gastric mucosa tissue and (b) gastric cancer tissue from the same patient. FAF1 staining localized mainly to the nucleus and in some cases to the cell membrane. Magnification, 200x.

Some authors have reported longer survival in Fas-positive cancer patients, suggesting that high Fas expression may inhibit tumor growth and implying that malfunction of Fas-dependent apoptotic pathways may be associated with more aggressive tumor formation and shorter survival [21]. FAF1 is a Fas-binding protein that negatively regulates capsaicin-induced apoptosis of cancer cells [22]. In the present study, we found that expression of both FAF1 mRNA and protein was reduced in gastric cancer tissue, and the level of FAF1 mRNA expression was associated with tumor differentiation, distant metastasis, and clinical stage of tumors.

Bjørling-Poulsen et al. [9] used Western blotting to show that levels of FAF1 protein were lower in gastric cancer tissue than in noncancerous tissue. Since protein levels are only an indirect measure of gene expression, we wished to probe directly whether FAF1 gene expression was altered during gastric cancer. Therefore we carried out RT-PCR studies in combination with immunohistochemistry to gain a more comprehensive picture of gene and protein regulation. Our results confirm the work of Bjørling-Poulsen et al. [9]. They further establish RT-PCR detection of FAF1 mRNA as a reliable and sensitive technique that may prove useful as a novel diagnostic tool for gastric cancer.

While the role of FAF1 in apoptosis is well known, its involvement in cell differentiation is less clear. At the very least, it is expected to play a key role, since FAF1-deficient mouse embryos die around the 2-cell stage, indicating that FAF1 is essential for cell viability and/or cell division [23]. FAF1 has been reported to act as a negative regulator of Wnt/beta-catenin signaling, which plays an important role in the regulation of cell proliferation and differentiation [24]. Given the proapoptotic function of FAF1, downregulation would be expected to promote the survival of tumor cells as well as their resistance to anticancer therapy [6]. In fact, FAF1 has recently been shown to mediate chemotherapy-induced apoptosis by promoting the formation of death effector filaments (DEFs), structures associated with receptor-independent apoptosis [25]. These findings may mean that increasing FAF1 expression or activity would be a good therapeutic goal.

Metastasis is a complex process involving degradation of the basement membrane, invasion of the stroma, adhesion,

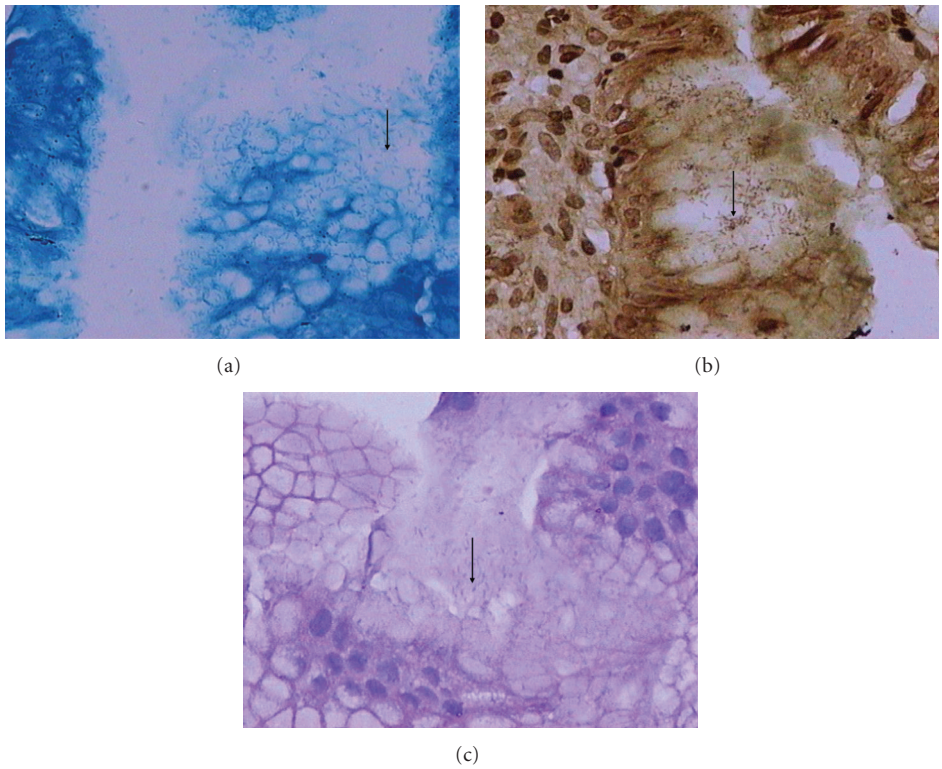


Figure 3: *H. pylori* aggregation on the surface of gastric mucosa. *H. pylori* was detected as (a) light blue staining using toluidine blue, (b) brownish-black staining using Warthin-Starry silver, and (c) light purple using H&E. Arrows indicate *H. pylori* bacilli. Magnification, 400x.

angiogenesis, and cell proliferation and migration. Our findings suggest that reduced FAF1 expression is associated with gastric cancer metastasis. This association may be related to recent research describing a role for miRNAs in biological pathways related to metastasis, including angiogenesis and apoptosis [26]. MiR-24 has been reported to target the FAF1 gene in the prostate cancer cell line DU-145 [27]. It would be interesting to investigate whether MiR-24 helps to explain the observed association between FAF1 gene downregulation and gastric cancer metastasis.

We also found FAF1 gene expression to be much lower in stage IV gastric cancer than in earlier stages of the disease. This may reflect the same processes behind the association between FAF1 downregulation and metastasis, since metastasis is indispensable for aggressive development of gastric cancer. The subgroup analyses by metastasis and clinical staging suggest that FAF1 acts as a tumor suppressor and its downregulation contributes to gastric cancer onset and progression. On the other hand, we found that FAF1 mRNA expression was higher in well-differentiated cancer tissue than in poorly differentiated cancer tissue. In addition, FAF1 mRNA expression showed no obvious association with tumor size, infiltration degree, lymph node metastasis or clinical stage below IV. It is possible that we failed to detect associations between FAF1 expression and clinicopathological features of gastric cancer because of our small sample size, in particular in our subgroup analyses, in which the smallest subgroup had only 3 patients. Therefore our findings require evaluation in future studies with larger sample sizes.

Various tools have been employed to identify the association between *H. pylori* and gastric cancer. Gene expression analysis using cDNA microarrays has shown that *H. pylori* infection significantly alters the expression of genes related to apoptosis and proliferation in human gastric carcinoma cells [28, 29]. However, we are unaware of reports probing the possible association between FAF1 gene expression and *H. pylori* infection in cancerous and noncancerous gastric mucosa. In our study, we used a strict triple-staining approach to detect *H. pylori* infection, which was found in 21 of 40 (52.5%) gastric cancer patients. This frequency is lower than the 171 of 214 cases (80%) reported by Motta et al. [30]. The reason for the discrepancy may be due to the smaller number of patients in our study, or to the fact that we applied a stricter screening method. These findings suggest that *H. pylori* infection may contribute to the downregulation of FAF1 gene expression during gastric carcinogenesis, and that this may contribute to malignant transformation. However, since a substantial percentage of our patients did not show the presence of *H. pylori*, our results suggest that the pathogen is not a prerequisite for onset or progression of gastric cancer.

Instead, the findings of our studies and others suggest that the development of gastric cancer is a multifactorial process in which *H. pylori* can participate but does not have to. Several studies have suggested plausible ways in which *H. pylori* infection may contribute to gastric cancer. The bacteria can induce Fas Ag- and ligand-mediated apoptosis, either directly or indirectly via cytokines IL-1β, TNFα and IFNγ

[31]. Increased apoptosis following *H. pylori* infection leads to gastric atrophy, while inhibition of apoptosis results in cell proliferation and transformation into cancer. If this occurs early during infection and persists, gastric cells can become resistant to Fas-mediated apoptosis [32, 33]. The Fas pathway may then drive cell proliferation and cell turnover. *H. pylori* can also bind to MHC-II in cultured cells and inhibit Fas Ag-mediated apoptosis [34]. In the present study, we found evidence for an additional effect of *H. pylori* infection: FAF1 mRNA expression was lower in *H. pylori*-positive tissue samples than in *H. pylori*-negative samples from gastric cancer patients. This result, moreover, was specific to cancerous tissue: the presence or absence of *H. pylori* did not affect the levels of FAF1 mRNA in noncancerous mucosa samples from the same gastric cancer patients. Our results suggest that *H. pylori* infection inhibits FAF1 mRNA expression, but this finding remains speculative. Future studies should examine larger samples sizes and should compare samples from gastric cancer patients and control samples from age- and sex-matched healthy individuals.

Our study was limited by a relatively small number of patients. In particular, subgroups were small, affecting the reliability of our analyses. In this study, we did not evaluate a possible association between FAF1 protein levels and *H. pylori* infection, which requires further study. Our work was also unable to identify how FAF1 activity may contribute to gastric cancer onset and progression. FAF1 has multiple protein-interacting domains and it may function in several signal transduction pathways [35, 36], so careful mechanistic studies are needed. We postulate that loss of FAF1 function may have far-reaching effects in cancer, although its role(s) may be context-dependent. Larger, more extensive studies are needed to understand better the role of FAF1 in signaling pathways vital to both normal development and tumorigenesis in *H. pylori* infection.

5. Conclusions

Expression of FAF1 mRNA and protein was lower in gastric cancer samples than in normal gastric mucosa samples from the same patients. Downregulation of FAF1 mRNA was associated with tumor differentiation and distant metastasis. *H. pylori* may downregulate FAF1 expression in gastric carcinogenesis. Consequently, increasing FAF1 expression through gene therapy may be effective for treating gastric cancer in the future.

Conflict of Interests

The authors declare that they have no conflict of interests.

Authors' Contribution

A.-q. Liu, L.-y. Ge, and X.-q. Ye contributed equally to this work.

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Research Article

Clinical Outcomes of the Marginal Ulcer Bleeding after Gastrectomy: As Compared to the Peptic Ulcer Bleeding with Nonoperated Stomach

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Background. Marginal ulcer is a well-known complication after gastrectomy. Its bleeding can be severe, but the severity has rarely been reported. We aim to evaluate the clinical outcomes of marginal ulcer bleeding (MUB) as compared to peptic ulcer bleeding (PUB) with nonoperated stomach. *Methods*. A consecutive series of patients who had nonvariceal upper gastrointestinal bleeding and admitted to the hospital between 2005 and 2011 were retrospectively analyzed. A total of 530 patients were enrolled in this study, and we compared the clinical characteristics between 70 patients with MUB and 460 patients with PUB. *Results*. Patients with MUB were older (mean age: 62.86 ± 10.59 years versus 53.33 ± 16.68 years, $P = 0.01$). The initial hemoglobin was lower (8.16 ± 3.05 g/dL versus 9.38 ± 2.49 g/dL, $P = 0.01$), and the duration of admission was longer in MUB (7.14 ± 4.10 days versus 5.90 ± 2.97 days, $P = 0.03$). After initial hemostasis, the rebleeding rate during admission was higher (16.2% versus 6.5%, $P = 0.01$) in MUB. However, the mortality rate did not differ statistically between MUB and PUB groups. *Helicobacter pylori*-positive rate with MUB was lower than that of PUB (19.4% versus 54.4%, $P = 0.01$). *Conclusions*. Clinically, MUB after gastrectomy is more severe than PUB with nonoperated stomach. Infection with *H. pylori* might not appear to play an important role in MUB after gastrectomy.

1. Introduction

Upper gastrointestinal bleeding remains a common emergency situation. Even though there have been significant improvements in endoscopic and supportive therapies, the overall mortality still remains around 10% and may even reach 35% in hospitalized patients with serious comorbidity [1]. Partial or total gastrectomy has been successfully performed in the treatment of peptic ulcer disease or gastric cancer; however, the potential complications after gastrectomy are numerous.

One of the major complications is bleeding from marginal ulcer [2]. Marginal ulcer is defined as an ulceration around gastroduodenal or gastrojejunal anastomosis site following partial gastric resection. Its incidence varies from 1% to 16%, and the etiology remains obscure [3, 4]. The possible contributing factors include local ischemia, anastomotic tension, increased gastric acidity, tobacco use, nonsteroidal anti-inflammatory drug use, and chronic irritation caused by the suture materials at the anastomosis [5–8]. However, only a few reports are available on the severity of marginal ulcer bleeding (MUB) after gastrectomy. Furthermore, it is well known that the etiology of peptic ulcer disease is the colonization of *Helicobacter pylori* (*H. pylori*) in the gastric mucosa, whereas the effect of this organism on the remnant stomach following gastrectomy still remains uncertain.

The aims of this study were to evaluate the differences in the clinical characteristics and the outcomes such as initial hemoglobin, duration of admission, rebleeding rate, and the rate of surgical treatment between MUB and peptic

ulcer bleeding (PUB) in patients with nonoperated stomach. Moreover, the association of MUB and *H. pylori* would be elucidated in patients with a history of gastrectomy.

2. Methods

2.1. Patients. The study was conducted at St. Vincent and St. Paul Hospital, the Catholic University of Korea. The medical records, charts, and the digitalized picture archived images of consecutive patients who were admitted for nonvariceal upper gastrointestinal bleeding between January 2005 and January 2011 were collected. Patients presented with objective evidence of upper gastrointestinal bleeding (hematemesis, melena, or blood from nasogastric aspirates).

All patients underwent an emergency esophagogastroduodenoscopy within 24 hours of initial presentation. No systemic sedative agent was given to any patient. The stigmata of bleeding were classified according to the Forrest classification (Ia, spurting bleeding; Ib, oozing bleeding; IIa, visible vessel; IIb, clot; IIc, black base; III, clear ulcer base) [9]. When the base of ulcer was classified as the Forrest classification I and IIa, the endoscopic treatment was done. In a small number of patients with Forrest IIb, endoscopic treatment was also performed. All patients had a second look endoscopy within 48 hours of initial endoscopic examination. During the second look endoscopy, two biopsy specimens were taken from the antrum (the greater curvature of the mid-antrum) and the corpus (the greater curvature of the midbody) for the histological assessment. The diagnosis of *H. pylori* infection was made by showing histological results—rapid urease test (CLO test, Kimberly-Clark, Utah, United States) or Warthin-Starry silver stain in any of two specimens from the antrum and body. If a case was reported as *H. pylori*-negative, a biopsy for the detection of *H. pylori* was repeated after 4~8 weeks. Alcohol consumption was defined as the consumption of at least 20 g alcohol/day and up to three times/week. Smoking was defined as current smoker.

A total of 530 patients had bleeding ulcers confirmed by endoscopy. Seventy patients had a history of gastrectomy. The outcome of this group of patients was compared to 460 patients without a history of gastrectomy. The patients excluded were all less than 17 years and older than 85 years of age. We excluded procedure-related bleeding (e.g., after gastric polypectomy, endoscopic mucosal resection, or endoscopic submucosal dissection) and patients with a medical comorbidity of serious systemic disease (heart failure, liver cirrhosis, chronic obstructive pulmonary disorder, sepsis, hematologic disorder, etc). However, the patients having diabetes mellitus without complication or hypertension with well-controlled state were included. Individuals with conditions that might have substantial effects on our study results (e.g., serum creatinine > 2.5 mg/dL and total bilirubin > 3.0 mg/dL), and a previous history of peptic ulcer disease, and bleeding associated with malignancy or nonulcer disease (varices, vascular ectasia, Dieulafoy's ulcer, Mallory-Weiss tear, and hemorrhagic erosive gastritis) were excluded. We also excluded the ulcers within first 1 year following gastrectomy.

2.2. Methods. The patient age, sex, smoking and alcohol history, initial hemoglobin, duration of admission, endoscopic findings, status of *H. pylori* infection, and clinical outcome were evaluated. Recurrence of bleeding was defined as the objective evidence of bleeding with continuous melena, hematochezia, or the presence of fresh bloody vomitus. When hemodynamic instability (systolic blood pressure <90 mmHg or heart rate >120 beats/minute) had developed or an abrupt drop of more than 2 g/dL of hemoglobin level occurred, these were also defined as a recurrence of bleeding.

2.3. Statistical Analysis. The continuous variables were expressed as a mean ± standard deviation and compared using the Student's *t*-test. The categorical variables were expressed as percentages and compared using a Chi-square test with SPSS version 12.0 software (SPSS Korea, Seoul, Korea). A *P*-value of less than 0.05 was regarded as significant.

2.4. Ethics Statement. This study was approved by the Institutional Review Board of the Catholic University of Korea (VC12RISI0015).

3. Results

Of the 70 patients with a history of gastrectomy, 33 were excluded due to a history of a gastrectomy within 1 year ($n = 8$), older than 85 years ($n = 3$), simple closure ($n = 2$), significant medical comorbidity ($n = 5$), and bleeding associated with malignancy ($n = 15$) (Figure 1). Of the 460 patients with a nonoperated stomach, 291 were excluded due to extreme age ($n = 24$), a significant medical comorbidity ($n = 155$), procedure-related bleeding ($n = 17$), repeated admissions for peptic ulcer disease ($n = 52$), bleeding from nonulcer disease ($n = 25$), and bleeding associated with malignancy ($n = 18$) (Figure 2). A total of 37 patients with MUB and 169 patients with PUB were enrolled. The characteristics and clinical outcomes of the patients of both groups are shown in Table 1.

The location of MUB with a history of gastrectomy was jejunal side in 20 patients (54.1%), anastomosis site in 12 (32.4%), and both sites in 5 (13.5%) (Figure 3). In PUB with nonoperated stomach, 93 patients (55.0%) had gastric ulcer bleeding, whereas 61 patients (36.1%) had duodenal ulcer bleeding. Fifteen patients (8.9%) had both gastric ulcer and duodenal ulcer bleeding.

A total of 24 patients were treated with a gastrectomy for the complications of peptic ulcer disease, whereas 12 patients had an operation for gastric cancer or a gastrointestinal stromal tumor. In the remaining one patient, the cause of gastrectomy was traumatic complication for traffic accident. Five patients of MUB had Billroth-I (B-I) gastroduodenal anastomosis and 32 patients had Billroth-II (B-II) gastrojejunal anastomosis (Table 2).

At the clinical aspect, the patients with MUB were older (mean age: 62.86 ± 10.59 years versus 53.33 ± 16.68 years, $P = 0.01$). The initial hemoglobin at presentation was lower (8.16 ± 3.05 g/dL versus 9.38 ± 2.49 g/dL, $P = 0.01$), and the duration of admission was longer in MUB (7.14 ± 4.10 days

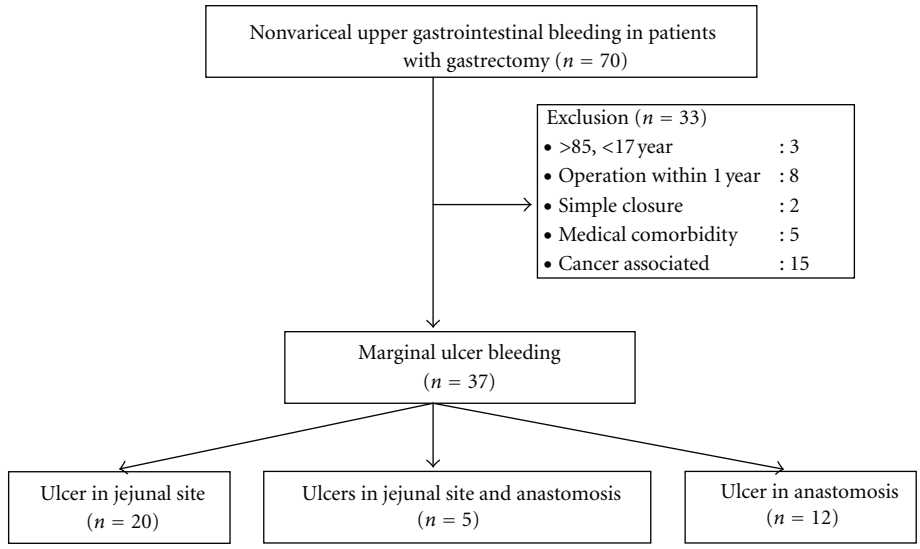
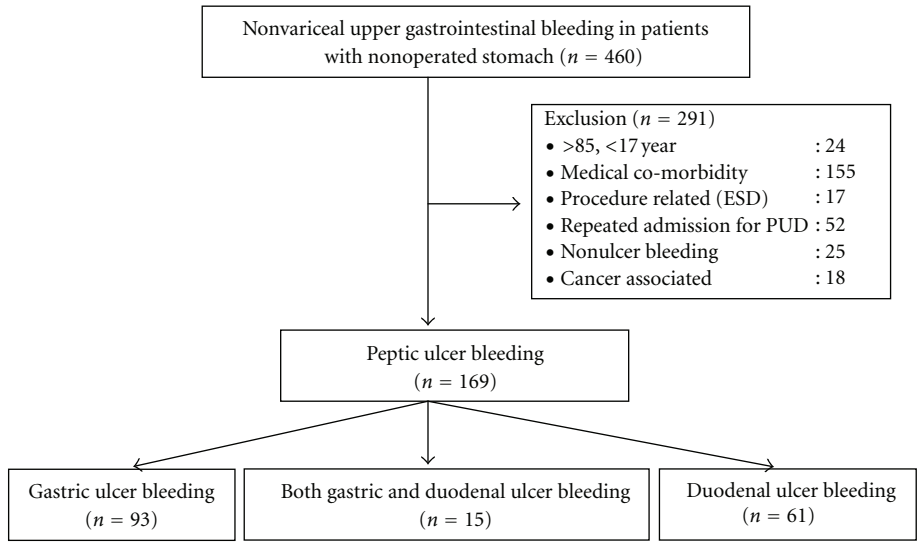


Figure 1: Study enrollment of the marginal ulcer bleeding after gastrectomy.



ESD: endoscopic submucosal dissection

PUD: peptic ulcer disease.

Figure 2: Study enrollment of the peptic ulcer bleeding with nonoperated stomach.

versus 5.90 ± 2.97 days, $P = 0.03$). At the initial endoscopic examination, definite bleeding stigmata (Forrest I and IIa) were identified in 15 patients (40.5%) with MUB, whereas 81 patients (47.9%) with PUB ($P = 0.40$). However, the frequency of therapeutic intervention was 37.8% (14/37) in MUB, whereas 56.8% (96/169) in PUB ($P = 0.03$). After an initial hemostasis, the rebleeding rate during admission was higher (16.2% versus 6.5%, $P = 0.01$) in MUB. However, the rate of surgical treatment did not differ statistically between MUB and PUB groups (2.7% versus 4.1%, $P = 0.66$). The infection rates of *H. pylori* in PUB with nonoperated stomach were higher than MUB (54.4% versus 19.4%, $P = 0.01$) after gastrectomy.

According to the cause of gastrectomy, MUB was subclassified into complicated peptic ulcer group and nonulcer group. The MUB with a history of complicated ulcer is 64.9% (24/37). There were no differences of age, initial hemoglobin level, rebleeding rate at admission, and the duration of admission. There was male-predominant feature in MUB with complicated ulcer ($P = 0.02$). The frequency of *H. pylori* infection did not differ significantly between the two groups (Table 2).

Table 1: Characteristics and clinical outcomes of the patients.

	MUB after gastrectomy ($n = 37$)	PUB with non-operated stomach ($n = 169$)	P value
Age	62.86 ± 10.59	53.33 ± 16.68	0.01
Sex (M : F)	23 : 14	130 : 39	0.06
Smoking (yes : no)	18 : 19	81 : 88	0.93
Alcohol (yes : no)	12 : 25	42 : 127	0.15
Initial hemoglobin (g/dL)	8.16 ± 3.05	9.38 ± 2.49	0.01
Duration of admission (day)	7.14 ± 4.10	5.90 ± 2.97	0.03
H. pylori infection (%)	9 (19.4)	93 (54.4)	0.01
Ulcer size (cm)	0.84 ± 0.57	1.00 ± 0.71	0.20
Multiplicity of ulcer (%)	16 (43.2)	44 (26.0)	0.04
Ulcer base (Forrest I, IIa) (%)	15 (40.5)	81 (47.9)	0.40
Therapeutic intervention (%)	14 (37.8)	96 (56.8)	0.03
Rebleeding case (%)	6 (16.2)	11 (6.5)	0.01
Surgical treatment (%)	1 (2.7)	7 (4.1)	0.66

Values shown as mean ± SD or No.

Table 2: Characteristics and clinical outcomes of MUB according to the history of a peptic ulcer.

	MUB with history of complicated ulcer ($n = 24$)	MUB without history of ulcer ($n = 13$)	P value
Age	61.54 ± 12.46	66.69 ± 7.25	0.30
Sex (M : F)	18 : 6	5 : 8	0.02
Smoking (yes : no)	12 : 12	4 : 9	0.25
Alcohol (yes : no)	9 : 15	3 : 10	0.37
Anastomosis			
Billroth-I	3	2	0.81
Billroth-II	21	11	
Initial hemoglobin (g/dL)	8.31 ± 2.10	7.84 ± 3.04	0.68
Duration of admission (day)	6.96 ± 2.70	6.85 ± 2.01	0.93
H. pylori Infection (%)	6 (25)	3 (23.1)	0.89
Rebleeding case (%)	3 (12.5)	3 (23.1)	0.40

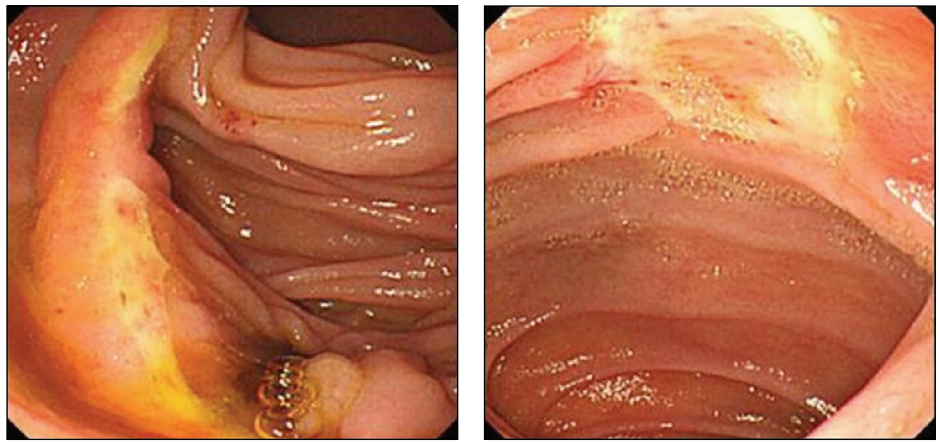
Values shown as mean ± SD or No.

4. Discussion

In this study, MUB after a gastrectomy seems to be more severe than PUB with non-operated stomach. This study was unique in three ways. In the first place, we focused on bleeding from benign peptic ulcer (including marginal ulcer), excluding bleeding from malignancy, Mallory-Weiss tear, vascular ectasia, angiodysplasia, and Dieulafoy's ulcer. Secondly, we excluded the patient group of medical comorbidity with systemic disease and extremely old age, because those factors might influence the outcomes of bleeding ulcer. Thirdly, we analyzed the status of *H. pylori* infection with the repeated histological examination.

Previously, it was reported that upper gastrointestinal bleeding would be more severe in surgically treated patients than in nonoperated patients [10]. Although it was a large scaled comparative study, the patients with extremely age and with significant concurrent diseases were not excluded. It would create biased results; therefore, we made concrete criteria to enroll the patients to avoid a bias in this study. Moreover, MUB within 1 year of gastrectomy was excluded from our study. In earlier study, there was a high incidence of marginal ulcer 1 month after surgery (early marginal ulcer), whereas a very low incidence 1 or 2 years after surgery (late marginal ulcer) [11]. It was suggested that different etiological factors were involved in the development of marginal ulcer after gastrectomy. Chronic irritation caused by the suture materials at the anastomosis, use of electrocautery, ischemic injury, and anastomotic stricture may lead to early marginal ulcer formation [4, 8, 11–13]. Although the incidence of late marginal ulcer was low, high output of gastric acid might be a main pathogenesis [12–15]. Therefore, we included only ulcers that developed 1 year or more after surgery, and true marginal ulcer after a gastrectomy that related to the gastric acid could be analyzed.

Since the discovery of *H. pylori*, epidemiologic and clinical studies have provided convincing evidence that *H. pylori* infection is the cause of peptic ulcer disease. It has been accepted that *H. pylori* is a major cause of peptic ulcer [16, 17]. However, in some cases, miscellaneous causes developed peptic ulcer disease without *H. pylori* infection. Until now, it is believed that an imbalance between the protective and aggressive factors acting on the mucosa plays a decisive role in the pathogenesis of a peptic ulcer. Aging and



(a) (b)

Figure 3: Representative images of the marginal ulcer bleeding in a patient with subtotal gastrectomy. (a) A linear ulceration was observed at anastomosis site. (b) A round ulcer was found on efferent side of Billroth-II anastomosis. And there were red spots on the base of ulcer.

male sex are also considered as risk factors for peptic ulcer disease [18–20]. Our results revealed that patients with MUB were older than PUB with nonoperated stomach. The male-dominant pattern in MUB was less typical than PUB, but there was no statistically significant difference. When MUB was subclassified into complicated ulcer group and nonulcer group, MUB with a history of complicated ulcer was two-thirds of total patients. Except the male-predominant pattern in the ulcer group, there were no differences in the variables for the severity of ulcer bleeding—initial hemoglobin level and duration of admission. In general, the prevalence of peptic ulcers in male patients is markedly higher than that of female patients. This sex difference is still seen in MUB with a history of complicated ulcer. The mechanism by which men have a higher prevalence of peptic ulcer is not clear. Possible mechanisms are suggested as high smoking rate and higher capacity of acid secretion in men. Another possible explanation is that female sex hormones may prevent the development of ulcer. Regretfully, there is no convincing evidence of male preference in ulcer disease.

Although the reason why MUB after gastrectomy has more severe clinical course remains unclear, it may be related to the iron deficiency anemia, postoperative changes of blood supply, and adhesion with fibrosis of the anastomosis site. The marginal ulcers in Billroth II anastomosis do not have the same mucosa or blood supply system as a peptic ulcer. The marginal ulcer receives blood supply from the jejunal branches of the superior mesenteric artery or gastric branches of the celiac artery [21]. Most of the marginal ulcers are found at the saddle area of the jejunum, and it made angulation among the arteries. The angulation phenomenon supplied the minimum requirement of blood to the mucosal fold of the jejunal saddle area [21]. Moreover, the saddle area of the jejunal loop is the mechanical weak point during food impulsion [10]. The impaired blood flow and the mechanical weak point may partially break the defense mechanism of the jejunal mucosa after injury [2]. Postoperative adhesion with fibrosis just and around the anastomosis site may influence blood supply of a bleeding ulcer site and the ulcer healing mechanism. In our results, more than half of marginal ulcer bleeding were at jejunal side.

Previously, it has been reported that the presence of a spurting artery is noted more commonly in patients having a history of gastric surgery [10]. However, in our study there was no difference of frequencies of recent bleeding stigmata of ulcer base (Forrest classification I and IIa) between MUB and PUB with nonoperated stomach. It was noteworthy that the frequency of therapeutic intervention in MUB was lower, but the rebleeding rate was higher than PUB with nonoperated stomach. The prediction of rebleeding based on Forrest classification might have a limitation in patients with MUB after gastrectomy, and it needs to be confirmed through further study. A new system for risk stratification of rebleeding would be proposed, and this system might consider both the clinical factors and endoscopic signs in MUB.

The exact link between *H. pylori* infection and MUB after a gastrectomy is unclear, and *H. pylori* infection is not associated with the severity of MUB. Previously, uncontrolled data have suggested that the frequency of marginal ulcers can be reduced by preoperative screening and treatment of *H. pylori* infection in patients undergoing gastric bypass surgery. This indicates that infection with *H. pylori* may promote marginal ulcer formation [22, 23]. On the contrary, several investigations revealed that the rate of *H. pylori* infection did not play an important role in the pathogenesis of marginal ulcer [24, 25]. In this study, the infection rate of *H. pylori* was significantly low in MUB. However, we could not rule out the possibility that some cases were negative for *H. pylori* at the time of diagnosis of MUB but had suffered from *H. pylori* infection previously. Most of the patients had partial gastrectomy with B-II anastomosis. Bile reflux may result in the interference with colonization by *H. pylori* and spontaneous clearance of the infection. This phenomenon is more typical in B-II than B-I anastomosis [26, 27]. Moreover, biopsy sites suitable for

the diagnosis of *H. pylori* infection in the operated stomach have yet to be decided. The potential limitation of our study was the retrospective design; therefore, the possible bias was inherent. Further research with prospective design in this area would be needed.

In conclusion, MUB in the patients with a history of gastrectomy is more severe than PUB with nonoperated stomach. In contrast with PUB, the association of MUB and the infection rate of *H. pylori* is low.

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