Association of epithelial and subepithelial *H. pylori* distribution detected by immunohistochemistry in gastric biopsies and clinical outcomes of patients receiving proton pump inhibitors

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Abstract. Studies have shown that *Helicobacter pylori* (*H. pylori)* can penetrate both the epithelium and subepithelium, which may lead to underdiagnosis. Rod-shaped *H. pylori* can transform into coccoid forms during proton pump inhibitor (PPI) therapy. However, the relationships among PPI use, bacterial invasiveness and clinical outcomes remain unclear. In the present retrospective study, the relationships among PPI use, *H. pylori* distribution patterns and clinical outcomes were investigated. Gastric tissue biopsies obtained between October and December 2022 were retrospectively studied using BioGenex immunohistochemical antibodies. *H. pylori* distribution was graded on the basis of surface epithelial and subepithelial locations. The overall pattern of *H. pylori* infection was determined from the grade and pattern of distribution. Data from patients with and without PPI use were compared. Among the 255 patients, those with and without PPI use had significantly different isolated epithelial patterns. Multivariate analysis revealed a positive correlation between PPI use and the isolated surface epithelial pattern and an inverse correlation with the isolated subepithelial pattern. Survival analysis revealed that the isolated subepithelial pattern was associated with the poorest clinical outcomes. Furthermore, eradication rates were lower in patients with the isolated subepithelial pattern than in patients with isolated surface epithelial pattern or other mixed patterns. The results of the present study suggested that PPI use altered the gastric mucosal microenvironment and bacterial biology. The presence of subepithelial *H. pylori* could be incorporated into pathological reports as ancillary studies along with the updated Sydney reporting system and could be incorporated into future clinical management.

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Introduction

Helicobacter pylori (H. pylori) is a highly prevalent infectious agent that affects ~40% of the global population (1). *H. pylori* was first discovered in 1983 by Robin Warren and Barry Marshall in gastric mucosal biopsy samples from patients with chronic active gastritis and peptic ulcer disease (2). Currently, this organism is recognized as the causative agent of gastritis, peptic ulcer disease, gastric adenocarcinoma and gastric B-cell lymphoma (MALT lymphoma) (3).

Several *in vivo* and *in vitro* studies have demonstrated that *H. pylori* can invade epithelial cells and the lamina propria, triggering a more intense mucosal inflammatory response than bacterial attachment to epithelial cells alone. This process is important in the initiation and development of gastric inflammation (4-7). Furthermore, *H. pylori* that invades the gastric mucosa may translocate to gastric lymph nodes, leading to chronic immune system stimulation (8).

H. pylori in the lamina propria or subepithelial regions may be underdiagnosed without an appropriate immunohistochemical (IHC) study. Ito *et al* (8) found *H. pylori* in both the mucous and deeper layers of gastric tissue in patients with gastric cancer using the TMDU antibody. However, the study focused on cancerous conditions and did not provide further descriptions or categorizations of the distribution patterns of the bacteria.

Proton pump inhibitors (PPIs) are among the most frequently prescribed medications to alleviate dyspeptic pain and have demonstrated *in vitro* anti-*H. pylori* activity (9). These drugs can reduce the *H. pylori* load while inhibiting its urease activity (10,11) and may interfere with certain *H. pylori* detection tests, potentially leading to false-negative results (12).

Following PPI therapy, rod-shaped *H. pylori* can convert into its coccoid form (13,14). Research has indicated that coccoid *H. pylori* may contribute to bacterial transmission and the recurrence of infection, even following antimicrobial treatment (13-16). However, the exact mechanisms underlying the pathogenesis of coccoid *H. pylori* remain poorly understood.

The detection of coccoid *H. pylori* requires IHC staining because simple histochemical stains cannot reliably detect low numbers of coccoid or intracellular bacteria, nor can they differentiate coccoid *H. pylori* from artefacts or other bacteria. IHC stains offer high specificity, enabling accurate

identification of *H. pylori* and exclusion of other similar-shaped organisms (17).

It was hypothesized that PPI treatment for acid suppression may modify the gastric mucosal microenvironment, influencing the distribution of *H. pylori* in both the surface and subepithelial compartments. Using IHC staining, it was aimed to identify distinct infection patterns and their potential associations with clinical outcomes.

Materials and methods

The study protocol complied with the principles of the Declaration of Helsinki and was approved by the Institutional Ethics Committee of Navamindradhiraj University (approval no. 120/2566; Bangkok, Thailand). Gastric tissue biopsies from patients diagnosed with gastritis, who underwent endoscopic examination at Vajira Hospital (Faculty of Medicine, Navamindradhiraj University, Bangkok, Thailand) between October and December 2022, were reviewed retrospectively.

Inclusion criteria were as follows: i) Availability of at least four biopsies obtained from both the antrum and corpus as part of routine clinical practice; ii) diagnosis of gastritis confirmed by histopathological evaluation; and iii) sufficient formalin-fixed paraffin-embedded (FFPE) tissue for both hematoxylin and eosin (H&E) and IHC analysis. Additional exclusion criteria included: vii) inadequate biopsy samples, and viii) poor tissue preservation precluding histological interpretation.

As part of standard clinical practice, at least four biopsies were collected from both the gastric antrum and corpus. Patients were not specifically recruited for the present study, and no additional tissue samples were collected. FFPE blocks were processed for histopathological and IHC analysis. Clinical data were also extracted and recorded between April and May 2024. All identifying information was removed.

The patients were divided into two groups according to PPI therapy: Those receiving PPIs for at least a 14-day period prior to the procedure (PPI-treated group) and those not receiving PPIs or receiving them for a shorter period (PPI-untreated group).

Exclusion criteria. Patients were excluded from the analysis on the basis of the following criteria: i) Previous use of antibiotics, histamine (H2) receptor blockers, statins, aspirin, or non-steroidal anti-inflammatory drugs; ii) a prior history of *H. pylori* infection or triple therapy eradication; iii) the presence of underlying conditions, including autoimmune diseases, diabetes mellitus, cerebrovascular disease, peptic ulcers, HIV infection, or gastrointestinal cancers; iv) use of immunosuppressive drugs prior to endoscopy; v) diagnosis of *Helicobacter heilmannii* infection based on morphological criteria (for example, straight appearance, corkscrew-shaped spirals, or exceeding 3 μm in length) (18); and vi) cases of gastritis with specific etiologies other than *H. pylori* infection, such as reactive gastropathy or autoimmune gastritis.

H. pylori eradication therapy. H. pylori eradication treatment was defined as the prescription of either a PPI with at least two types of antibiotics initiated simultaneously or a fixed-dose triple therapy. Eradication was considered successful if no second treatment was prescribed after the initial treatment. Treatment failure, defined as the prescription of a second eradication treatment within 12 months, was used as a proxy for antimicrobial resistance.

Histopathology. All the paraffin blocks were sectioned at 3-μm thickness and stained with H&E in August 2023. Staining was performed at room temperature (~22-25˚C), with hematoxylin applied for 5-10 min and eosin for 1-2 min, following standard histological protocols.

Two pathologists (CS and KL) independently reviewed the H&E-stained slides. Cases with discordant results were reviewed together and discussed until consensus was reached. Neutrophilic and mononuclear inflammation, glandular atrophy, and intestinal metaplasia were assessed and graded (0=none, 1=mild, 2=moderate, 3=severe) according to the updated Sydney System (19).

Gastritis was classified on the basis of inflammatory process status (chronic and/or active) and the presence or absence of structural changes (intestinal metaplasia, atrophy, ulcers). This resulted in four distinct groups: i) Chronic nonactive gastritis without structural change, ii) chronic active gastritis without structural change, iii) chronic non-active gastritis with structural change, and iv) chronic active gastritis with structural change.

To analyze the associations between pathologic features and *H. pylori* presence, the cases were further regrouped as 'non-active gastritis' (groups i and iii) and 'active gastritis' (groups ii and iv). Additionally, they were regrouped as 'without structural change' (groups i and ii) and 'with structural change' (groups iii and iv).

IHC. IHC tests were performed on all cases in August 2023 using the Leica Bond-Max automated IHC staining platform (Leica Microsystems, Inc.) with 2-μm-thick consecutive sections prepared from FFPE tissue, in accordance with the manufacturer's instructions. Tissue sections were deparaffinized using Leica Bond Dewax Solution (cat. no. AR9222), and rehydration was performed automatically by the Bond-Max system using a graded ethanol series integrated into the protocol. Antigen retrieval was conducted using Epitope Retrieval Solution 2 (ER2; pH 9.0; cat. no. AR9640) for 20 min at 100˚C under standard automated conditions.

Endogenous peroxidase activity was blocked using the Peroxidase Block reagent included in the Leica Bond Polymer Refine Detection kit (cat. no. DS9800), which contains ~3% hydrogen peroxide. This blocking step was carried out for 5 min at room temperature (~22-25˚), according to the system's default protocol.

The following primary antibodies targeting *H. pylori* were used: mouse monoclonal anti-*H. pylori* antibody (1:800; clone TMDU-D8; cat. no. D369-3; MBL International Co.), monoclonal anti-*H. pylori* antibody (1:300; clone ULC3R; cat. no. MU880-5UCE; BioGenex Laboratories), polyclonal anti-*H. pylori* antibody (1:100; cat. no. 215A-76; Cell Marque), and polyclonal anti-*H. pylori* antibody (1:50; cat. no. B0471; Dako; Agilent Technologies, Inc.). Primary antibody incubation was performed at room temperature for 15 min, consistent with Leica's standard program for the

Table I. Classification of 255 patients according to age, sex, histopathological findings, and *H. pylori* status by BioGenex (n=255).

Variables	Total, (n=255)		PPI positive (n=115)		PPI negative (n=140)		P-value
Age, years							
<60	159	(62.4)	68	(59.1)	91	(65.0)	
≥60	96	(37.6)	47	(40.9)	49	(35.0)	0.336[a]
Sex, n (%)							
Female	130	(51.0)	66	(57.4)	64	(45.7)	0.063[a]
Male	125	(49.0)	49	(42.6)	76	(54.3)	
Histology							
Chronic active gastritis with structural change	10	(3.9)	3	(2.6)	7	(5.0)	0.519[b]
Chronic non-active gastritis with structural change	40	(15.7)	20	(17.4)	20	(14.3)	0.497[a]
Chronic active gastritis without structural change	31	(12.2)	11	(9.6)	20	(14.3)	0.251[a]
Chronic non-active gastritis without structural change	174	(68.2)	81	(70.4)	93	(66.4)	0.494[a]
H. pylori status							
H. pylori positive	83	(32.5)	31	(27.0)	52	(37.1)	0.107[a]
Any surface	60	(23.5)	26	(22.6)	34	(24.3)	0.753[a]
Any subepithelium	75	(29.4)	25	(21.7)	50	(35.7)	0.015[a]

Data are presented as number, (%). P-value corresponds to [a]Chi-square test or [b]Fisher's exact test. PPI, proton pump inhibitors; *H. pylori*, *Helicobacter pylori*.

Bond-Max system. BioGenex demonstrated superior diagnostic performance for *H. pylori* detection and was therefore used as the reference standard (gold standard) in the present study (Table SI).

Immunoreactivity was visualized using the Leica Bond Polymer Refine Detection kit (cat. no. DS9800), with incubation performed at room temperature for 15-30 min, as per the manufacturer's protocol. Positive (*H. pylori*-infected gastric biopsy) and negative (gastric tissue from sleeve gastrectomy patients without gastritis) controls were included. Stained slides were examined using a Nikon Eclipse E200 light microscope.

Detection of H. pylori. Two pathologists (CS and KL) independently evaluated the *H. pylori* IHC slides. Disagreements were resolved through joint discussion until a consensus was reached. Bacterial density was assessed separately for surface epithelial and subepithelial locations. The surface epithelial *H. pylori* density was graded using the updated Sydney System's standardized visual analogue scale, which classifies bacteria into four categories (19): S0: normal (no bacteria); S1: individual bacteria or small groups of <1/3 of the mucosal surface; S2: moderate (bacteria count greater than mild but less than severe); S3: severe (large groups of bacteria covering >2/3 of the mucosal surface).

The subepithelial *H. pylori* location (either intracellular or interstitial) was examined under high magnification (0.196 mm^2 area, high-power field) using a Nikon Eclipse E200 light microscope. Subepithelial signals were counted below the lower border of the basement membrane, which served as the histological landmark.

For signal selection, only those exhibiting an intensity comparable to surface-attached bacilli morphology in the external or internal positive controls and showing a negative signal in the negative control were included. Homogeneous, indiscrete clumps were counted as a single signal, whereas heterogeneous, discrete signals within clumps were counted individually.

When the signal density varied across the slide, the region with the highest density (hot spot) was designated for counting. The subepithelial bacterial density was then graded on the basis of the number of immunopositive signals: I0: no signal; I1: 1-20 signals/HPF; I2: 20-50 signals/HPF; and I3: >50 signals/HPF.

Patterns of H. pylori. The antibodies demonstrating the highest diagnostic accuracy served as the gold standard for classifying *H. pylori* patterns. The classification, derived from combining bacterial presence in both surface epithelial and subepithelial locations, resulted in the following five groups: Group 1: Isolated surface epithelial pattern (S1-S3 and I0); Group 2: Isolated subepithelial pattern (I1-I3 and S0); Group 3: Predominant surface epithelial pattern (S3 and only I1); Group 4: Predominant subepithelial pattern (I3 and only S1); Group 5: Non-specific pattern (S1 and I1-I2, S2 and I1-I3, or S3 and I2-I3).

Statistical analyses. All the statistical analyses were conducted using Stata (version 13; StataCorp LP). The diagnostic performance of each IHC study was assessed by calculating the sensitivity, specificity, positive predictive value and negative predictive value.

Categorical variables were compared using the chi-square test or Fisher's exact test, as appropriate. Univariate and multivariate analyses utilized the Cox proportional hazards model. The time to clinical improvement (cumulative clinical

Table II. Comparison of histopathological characteristics and group classification between PPI and non-PPI users in the *H. pylori*-positive cases, using BioGenex as the gold standard.

Variables	Total (n=83)		PPI positive (n=31)		PPI negative (n=52)		P-value
Histology							
Chronic active gastritis with morphological change	10	(12.0)	3	(9.7)	7	(13.5)	0.737[b]
Chronic nonactive gastritis with morphological change	17	(20.5)	8	(25.8)	9	(17.3)	0.353[a]
Chronic active gastritis without morphological change	26	(31.3)	10	(32.3)	16	(30.8)	0.888[a]
Chronic nonactive gastritis without morphological change	30	(36.1)	10	(32.3)	20	(38.5)	0.569[a]
HP positive	83	(100)	31	(100)	52	(100)	NA
Any surface staining	60	(72.3)	26	(83.9)	34	(65.4)	0.069[a]
Any subepithelial staining	75	(90.4)	25	(80.6)	50	(96.2)	0.047[b]
Isolated surface epithelial pattern	8	(9.6)	6	(19.4)	2	(3.8)	0.047[b]
Isolated subepithelial pattern	23	(27.7)	5	(16.1)	18	(34.6)	0.069a
Predominant surface epithelial pattern	17	(20.5)	7	(22.6)	10	(19.2)	0.879[a]
Predominant subepithelial pattern	8	(9.6)	2	(6.5)	6	(11.5)	0.704[b]
Non-specific pattern	27	(32.5)	11	(35.5)	16	(30.8)	0.657[a]

P-value corresponds to [a]Chi-square test or [b]Fisher's exact test. PPI, proton pump inhibitors; NA, not applicable.

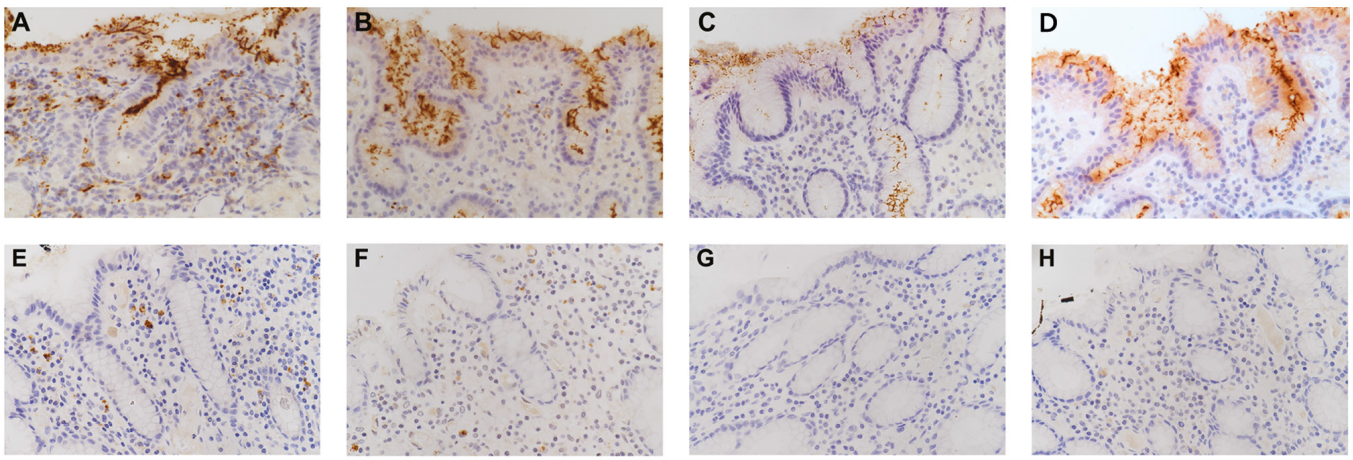

Figure 1. Topographic distribution of immunoreactive *H. pylori* according to surface epithelial and subepithelial locations, using BioGenex, TMDU, Cell Marque and DAKO. Although the detection frequency and staining intensity varied, the immunoreactive *H. pylori* did not differ morphologically among these antibodies when detected in the surface epithelium. Remarkable differences were observed in the IHC results for subepithelial *H. pylori* in the lamina propria. (A-D) Scattered dot-like particles were found with (A) BioGenex and, to lesser extent, with (B) TMDU, a few particles with (C) Cell Marque, and no reactivity with (D) DAKO. (E-H) In the isolated subepithelial pattern, (E) BioGenex and (F) TMDU identified subepithelial bacteria while (G) Cell Marque and (H) DAKO did not identify any bacteria. This also clarifies that all four markers were negative for surface bacteria (original magnification, x60). *H. pylori*, *Helicobacter pylori*.

improvement rate) was calculated as the duration between the onset of upper abdominal symptoms at diagnosis and either clinical improvement or the last follow-up. These data were compared between groups via Kaplan-Meier plots and the log-rank test.

To assess the associations between *H. pylori* infection patterns and treatment success, modified Poisson regression was used to estimate the relative risk (RR), with robust standard errors used to calculate 95% confidence intervals (CIs). Statistically significant difference was set at $P<0.05$. Only patients with consistent follow-up visits every 1-2 months for at least 6 months were included in the analysis.

Results

Demographic and histopathological characteristics of the study population. The present study included 255 patients with chronic gastritis diagnosed from tissue biopsies. The mean age of the patients was 47 years, and 130 (51.0%) were female. Nearly half (115 patients, 45.1%) had received PPI therapy, with a median treatment duration of 2.7 years. There were no significant differences in age or sex between the PPI-treated and untreated groups.

The demographic and histopathological characteristics of the study population are summarized in Table I. Among all

Table III. Correlation between *H. pylori*-positive cases and histologic grades according to the updated Sydney System, using BioGenex as the gold standard.

	Surface		Subepithelium	
Sydney system	+	-	+	-
Chronic inflammation				
0	0	0	0	0
1	0	0	0	0
2	4	1	2	3
3	22	4	23	3
r[a]	0.046		0.451	
P-value	0.805		0.011	
PMN infiltration				
0	13	5	13	5
1	0	0	0	0
2	3	0	2	1
3	10	0	10	0
r[a]	0.363		0.296	
P-value	0.045		0.105	
Glandular atrophy				
0	18	3	18	3
1	4	1	3	2
2	3	1	3	1
3	1	0	1	0
r[a]	-0.065		-0.149	
P-value	0.727		0.424	
Intestinal metaplasia				
0	22	3	20	5
1	0	1	1	0
2	4	1	4	1
3	0	0	0	0
r[a]	-0.200		0.027	
P	0.281		0.887	
H. pylori density				
Surface				
0	0	5	5	0
1	11	0	5	6
2	7	0	7	0
3	8	0	8	0
r[a]	0.663		0.285	
P-value	<0.001		0.120	
Subepithelium				
0	6	0	0	6
1	10	4	14	0
2	6	1	7	0
3	4	0	4	0
r[a]	0.021		0.727	
P-value	0.912		<0.001	

[a]Spearman's rank correlation coefficient (r). *H. pylori*, *Helicobacter pylori*.

participants, the most common type of gastritis, considering both inflammatory activity and structural changes, was chronic non-active gastritis without structural changes (68.2%). This was followed by chronic nonactive gastritis with structural changes (15.7%), chronic active gastritis without structural changes (12.2%), and chronic active gastritis with structural changes (3.9%). These trends were similar in patients receiving PPI treatment. However, among patients not using PPIs, chronic nonactive gastritis without structural changes was most common (66.4%). Chronic non-active gastritis with structural changes and chronic active gastritis without structural changes were equally prevalent (14.3%), whereas chronic active gastritis with structural changes was the least common (5.0%).

H. pylori detection rate. Among the 255 gastric biopsies, the *H. pylori* detection rate by BioGenex was 32.5%. Bacteria were detected in the surface epithelium in 23.5% of the cases (72.3% of positive cases) and in the subepithelial location in 29.4% of the cases (90.4% of positive cases). The performance of all four antibodies is illustrated in Fig. 1.

PPI use and H. pylori detection. A significantly lower *H. pylori* detection rate was observed among patients not using PPIs than among those in the PPI-treated groups: 21.7% vs. 35.7% for overall subepithelial staining (P=0.015) and 3.8% vs. 19.4% for the isolated epithelial pattern (P=0.047), respectively. However, no statistically significant associations were found between PPI treatment and other mixed histopathology patterns of gastritis. The results are summarized in Table II.

Mucosal density and location of H. pylori and grades based on the Sydney system visual analogue scale. Using BioGenex as a reference, a weak correlation (r=0.36) was observed between the surface epithelial density of *H. pylori* (evaluated via IHC) and active inflammation in the epithelial compartment (gastric mucosa) (Table III). However, *H. pylori* density was moderately correlated (r=0.45) with chronic inflammation and not correlated with active inflammation in the subepithelial compartment.

Factors predicting H. pylori distribution patterns. Multivariate analysis (Table IV) revealed that PPI use and a histologically active pattern were independently associated with the isolated surface epithelial pattern [hazard ratio (HR), 8.02 and 3.55, respectively] and inversely associated with the isolated subepithelial pattern (HR, 0.24 and 0.05, respectively). Additionally, a histologically active pattern was independently associated with the predominant surface epithelial pattern (HR, 5.18) and the non-specific pattern (HR, 3.12). No statistically significant associations were observed between PPI treatment (treated vs. untreated groups) and age, sex, or the histopathology of gastritis.

Clinical data and follow-up. Of the 255 patients included in the histopathological and IHC studies, 83 patients with *H. pylori* infection had complete clinical follow-up for a minimum of 3 months and were included in the outcome analyses.

Overall, 55.4% (46 patients) achieved clinical improvement (Table V). The clinical outcomes of patients on the basis of

Table IV. Multivariable analyses of factors associated with *Helicobacter pylori* distribution patterns.

	Multivariable analysis (G1)			Multivariable analysis (G2)			Multivariable analysis (G3)			Multivariable analysis (G4)			Multivariable analysis (G5)		
Variables	OR_{adj}[2]	95% CI	P-value	OR_{adj}[2]	95% CI	P-value	OR_{adj}[2]	95% CI	P-value	OR_{adj}[2]	95% CI	P-value	OR_{adj}[2]	95% CI	P-value
Age, years															
<60	1.00	Reference		1.00	Reference		1.00	Reference		1.00	Reference		1.00	Reference	
≥60	1.01	(0.34-3.02)	0.989	1.04	(0.32-3.33)	0.953	1.30	(0.41-4.06)	0.657	2.09	(0.38-11.56)	0.398	0.80	(0.30-2.12)	0.655
Sex															
Male	1.00	Reference		1.00	Reference		1.00	Reference		1.00	Reference		1.00	Reference	
Female	1.08	(0.38-3.12)	0.881	0.68	(0.21-2.16)	0.509	3.82	(1.25-11.68)	0.019	0.77	(0.16-3.65)	0.738	0.73	(0.28-1.89)	0.518
Treatment															
PPI negative	1.00	Reference		1.00	Reference		1.00	Reference		1.00	Reference		1.00	Reference	
PPI positive	8.02	(2.62-24.50)	<0.001	0.24	(0.07-0.84)	0.026	0.59	(0.19-1.86)	0.366	0.60	(0.11-3.40)	0.565	1.09	(0.41-2.89)	0.869
Histology															
Active inflammation															
Non-active pattern[a]	1.00	Reference		1.00	Reference		1.00	Reference		1.00	Reference		1.00	Reference	
Active pattern[a]	3.55	(1.18-10.72)	0.024	0.05	(0.01-0.25)	<0.001	5.18	(1.73-15.51)	0.003	0.36	(0.07-1.96)	0.237	3.12	(1.22-8.00)	0.018
Structural alteration															
No structural change[b]															
With structural change[b]															

[a]Regardless of structural changes; [b]Regardless of gastritis activity. NA, data not applicable; PPI, proton pump inhibitors; OR, odds ratio; OR_{adj}, adjusted OR; CI, confidence interval; Group 1 (G1), Isolated surface epithelial pattern; Group 2 (G2), isolated subepithelial pattern; Group 3 (G3), Predominant surface epithelial pattern; Group 4 (G4), Predominant subepithelial pattern; Group 5 (G5), Non-specific pattern.

Table V. Univariable analyses of the clinical improvement rate at 3 months in the *Helicobacter pylori*-positive cases according to various clinicopathologic factors (n=83).

			Clinical improvement rate at 3 months		
Variables	N	Number of improvements	Rate	(95% CI)	P-value
Total	83	46	55.42	(45.14-66.28)	
Age, years					
<60	31	14	45.16	(29.74-64.03)	0.474
≥60	52	32	61.54	(48.66-74.57)	
Sex					
Female	39	23	58.97	(44.24-74.31)	0.521
Male	44	23	52.27	(38.55-67.49)	
Treatment					
PPI negative	52	31	59.62	(46.74-72.89)	0.243
PPI positive	31	15	48.39	(32.64-66.96)	
Histopathology					
Active inflammation					
Non-active pattern[a]	47	17	36.17	(24.27-51.57)	
Active pattern[a]	36	29	80.56	(66.42-91.44)	0.005
Structural alteration					
No structural change[b]	56	31	55.36	(42.98-68.58)	
With structural change[b]	27	15	55.56	(38.25-74.44)	0.841
Topographic distribution					
Isolated surface or predominant surface epithelial or non-specific patterns	52	39	75.00	(62.74-85.73)	<0.001
Isolated subepithelial pattern	23	2	8.70	(2.25-30.51)	
Predominant subepithelial pattern	8	5	62.50	(32.56-91.3)	

Significant at P<0.05 (P-value corresponds to Log-rank test). [a]Regardless of structural changes; [b]Regardless of gastritis activity. CI, confidence interval; PPI, proton pump inhibitors.

the clinical and pathological features of gastritis, as well as the *H. pylori* pattern, are presented in Table VI. Univariable analysis revealed that patients with chronic gastritis with active inflammation experienced greater clinical improvement than those with non-active infections did (improvement rate: 80.6; 95% CI: 66.42-91.44; P=0.005), whereas patients with the isolated subepithelial pattern had the lowest improvement rate compared with the other groups (improvement rate: 8.7; 95% CI: 2.25-30.51; P<0.001). However, only the isolated subepithelial pattern was confirmed as an independent factor for clinical outcomes through multivariate analysis (HR, 0.25; 95% CI: 0.12-0.50; P<0.001). Notably, PPI treatment was not found to be associated with clinical outcomes. The Kaplan-Meier survival curves of patients according to their *H. pylori* distribution pattern is included in Fig. 2.

H. pylori eradication therapy. Among the *H. pylori*-positive patients, 73.5% (61 patients) received eradication therapy, while 26.5% (22 patients) were left untreated. Among the untreated patients, the majority (81.8%, 18 patients) had an isolated subepithelial pattern. Compared with the isolated surface epithelial pattern or mixed patterns, the isolated subepithelial pattern was associated with a lower eradication success rate. Modified Poisson regression analysis revealed that compared with the isolated subepithelial pattern, the surface epithelial pattern or mixed patterns had a greater risk ratio (RR) of successful *H. pylori* eradication, with RR values ranging from 1.46-1.67 (RR=1) (Table VII).

Discussion

In the present study, the prevalence of *H. pylori* infection, as determined by IHC in 255 patients, was 32.5%, which is lower than the 40-45% reported in previous studies (1,20,21). This discrepancy may be attributed to differences in study periods, with more recent research indicating a decline in prevalence compared with earlier studies (1,20,21). This reduction in infection rates could be due to demographic variations, improved personal hygiene, improved living conditions, increased public awareness, and active *H. pylori* eradication efforts (1,20,21).

The ability to detect *H. pylori* and its specific localization may vary depending on the antibody used for IHC staining. Certain antibodies typically detect *H. pylori* only in superficial areas and not beneath the epithelium. Ito *et al* (8) examined the presence and invasive behavior of *H. pylori* via various methods, including IHC, PCR, bacterial culture and immunoelectron

Table VI. Univariable and multivariable Cox proportional hazards regression survival analyses of factors associated with clinical improvement in *Helicobacter pylori*-positive cases.

	Univariable analysis			Multivariable analysis		
Variables	HR	95%CI	P-value	HR_{adj}	95%CI	P-value
Age (years)						
<60	1.00	Reference		1.00	Reference	
≥60	0.85	(0.54-1.35)	0.497	0.97	(0.58-1.63)	0.921
Sex						
Male	1.00	Reference		1.00	Reference	
Female	1.15	(0.74-1.78)	0.541	1.00	(0.64-1.57)	0.984
Treatment						
PPI negative	1.00	Reference		1.00	Reference	
PPI positive	1.30	(0.82-2.06)	0.268	0.78	(0.44-1.37)	0.388
Histopathology						
Active inflammation						
Non-active pattern[a]	0.55	(0.35-0.86)	0.009	1.13	(0.66-1.93)	0.664
Active pattern[a]	1.00	Reference		1.00	Reference	
Structural alteration						
No structural change[b]	1.00	Reference				
With structural change[b]	1.05	(0.66-1.66)	0.848			
Topographic distribution						
Isolated surface or predominant surface epithelial or non-specific patterns	1.00	Reference		1.00	Reference	
Isolated subepithelial pattern	0.30	(0.17-0.51)	<0.001	0.25	(0.12-0.50)	<0.001
Predominant subepithelial pattern	0.56	(0.26-1.22)	0.147	0.49	(0.22-1.12)	0.093

Significant at P<0.05 (P-value corresponds to Log-rank test). [a]Regardless of structural changes. [b]Regardless of gastritis activity; HR, hazard ratio; HR^{adj}, Adjusted HR; CI, confidence interval.

Table VII. Factors associated with the success rates of *H. pylori* eradication.

Patterns of *H. pylori*	Number of patients treated	Eradication rate (%)	Treatment failure (%)	RR	95% CI	P-value
Isolated surface epithelial pattern	8	100	0	1.67	(0.81-3.43)	0.165
Isolated subepithelial pattern	5	60	40	1.00	Reference	
Predominant surface epithelial pattern	15	93.3	6.7	1.56	(0.75-3.24)	0.238
Predominant subepithelial pattern	8	87.5	12.5	1.46	(0.68-3.14)	0.336
Non-specific pattern	25	92	8	1.53	(0.74-3.18)	0.252

H. pylori, *Helicobacter pylori*.

microscopy. They found *H. pylori* in both the mucous layer and the lamina propria in gastric cancer patients using the TMDU-mAb. In the present study, the diagnostic accuracy of BioGenex was comparable with that of TMDU, with BioGenex showing greater sensitivity in detecting *H. pylori* in deeper tissue, where TMDU was detected in only 68.9% of cases.

H. pylori was initially considered a non-invasive pathogen that resides solely in the gastric lumen and affects only gastric epithelial cells. However, most previous studies focused primarily on the surface epithelium. Subsequent *in vivo* and *in vitro* studies have shown that *H. pylori* is invasive and can also be found in the lamina propria (4-8). Expanding the focus to include the subepithelial location may provide a more accurate representation of *H. pylori* prevalence.

The presence of *H. pylori* in the subepithelial region correlated more strongly with the severity of chronic inflammation than surface bacteria in active gastritis, which aligns with the findings of Ito *et al* (8). This suggests a location-dependent

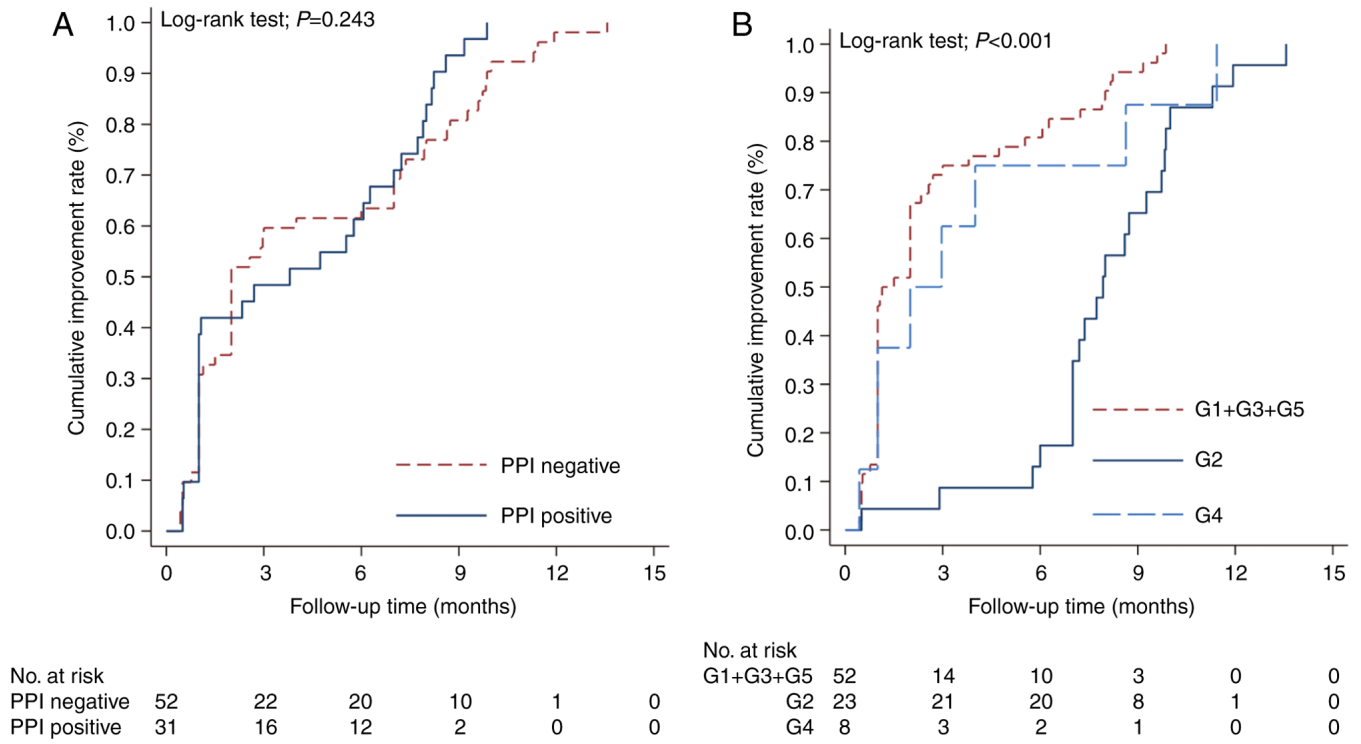

Figure 2. Kaplan-Meier survival curves for *H. pylori*-infected patients in relation to PPI use and bacterial distribution patterns. (A) Clinical improvement rate based on PPI users and non-users. (B) Clinical improvement rate based on group classification (G1 + G3 + G5 vs. G2 + G4). G1, surface epithelial pattern; G2, subepithelial pattern; G3, predominant surface epithelial pattern; G4, predominant subepithelial pattern; G5, non-specific pattern; PPI, proton pump inhibitors.

immunological response, potentially involving macrophage interaction and contributing to the induction and maintenance of chronic inflammation in *H. pylori*-infected gastric mucosa. While surface epithelial *H. pylori* typically induces IL-12 production and Th1 cell accumulation (22), subepithelial bacteria, interacting with lamina propria macrophages and T cells, may elicit a distinct chronic inflammation. The present study observed a significantly higher prevalence of isolated subepithelial *H. pylori* (27.7%) compared with Ito *et al*'s (2%). Notably, Ito *et al* (8) reported 46 PCR-positive *H. pylori* cases in the stomach; however, 4 of these cases exhibited negative IHC results for both surface and subepithelial bacteria, with only 1 showing isolated subepithelial positivity. The differences in findings may be explained by several factors. Firstly, Ito *et al* (8) employed more thorough tissue sampling, which may have led to an increased detection of surface bacteria.

In contrast to surface-attached bacilli, subepithelial *H. pylori* presented as dot-like signals, which may represent bacterial casts or coccoid forms. Additionally, the enhanced sensitivity of BioGenex used in the present study may explain the discrepancies. BioGenex utilizes the ULC3R clone to identify specific epitopes of *H. pylori* that are resistant to intracellular digestion and capable of detecting antigenic forms of *H. pylori* resembling the bacillary type, even in coccoid forms. BioGenex detected all TMDU-positive cases in the subepithelium, indicating greater sensitivity. Furthermore, differing positivity cutoffs (1 in the present study vs. ≥10 signals in Ito *et al*'s) could contribute to the observed variation. Consequently, the precise prevalence of isolated subepithelial *H. pylori* remains inconclusive, necessitating further validation.

A significantly lower detection rate of *H. pylori* in the subepithelial compartment was observed in patients treated with PPIs than in those not treated with PPIs. These findings suggest that PPI use may inhibit *H. pylori* infiltration into the lamina propria.

The underlying mechanism may involve inhibiting MMP-2/TIMP-3 interactions. These effects could protect against gastric mucosal injury, thereby reducing the degree of epithelial damage that facilitates bacterial translocation (23).

In addition to the diagnostic challenges of detecting subepithelial bacteria, their presence may have implications for clinical outcomes. First, subepithelial particles could signify residual bacteria from the surface that remain viable in unbiopsied areas of the stomach, potentially causing persistent symptoms. Second, immunoreactive signals in subepithelial regions may represent debris from dead bacteria, which can continue to trigger inflammation and contribute to ongoing inflammatory responses.

Third, subepithelial immunoreactive particles may reflect a transformation from the usual bacillary form of *H. pylori* to an atypical coccoid form under stressful conditions. These coccoid forms could revive and revert to culturable bacilli. This transformation may contribute to treatment failure and relapse, as coccoid forms may evade immune detection while remaining susceptible to antibiotics (13-16). These findings may explain our results, which revealed that the eradication rate of *H. pylori* treatment was greater in patients with isolated surface epithelial or mixed infection patterns than in those with an isolated subepithelial pattern. The presence of the latter pattern may provide indirect evidence supporting the consideration of a step-up eradication therapy regimen.

However, establishing a definitive link between the isolated subepithelial pattern and treatment success or failure remains challenging due to the small sample size.

Unlike treated patients, most untreated *H. pylori*-positive patients exhibited an isolated subepithelial pattern, likely due to underdiagnosis. Most treated patients are diagnosed using the rapid urease test (RUT), H&E staining, Giemsa, or polyclonal DAKO IHC, which are routinely employed in Vajira Hospital. By contrast, untreated patients predominantly presented with an isolated subepithelial pattern, which typically yields negative results for H&E, Giemsa, or polyclonal DAKO IHC. A total of 5 patients were treated on the basis of positive RUT results. However, RUT is rarely performed at our institution; treatment decisions are typically based on histopathology (H&E), special stains, or IHC results. This underscores the potential for significant underdiagnosis in real-world clinical practice.

Whether this staining represents live subepithelial bacteria or merely debris from dead bacteria remains unclear. Nevertheless, our results suggest that this immunoreactive signal should not be overlooked and warrants further investigation.

The current reporting system for *H. pylori* may benefit from the incorporation of immuno-stained subepithelial bacteria as a prognostic factor. The authors propose reporting *H. pylori*-IHC as an ancillary study alongside the Updated Sydney System to provide a clearer summary of *H. pylori* density and improve clinical communication. Specifically, infection patterns should be reported under the heading '*H. pylori*-positive cases', including specific location details. This would enable clinicians to accurately identify infection types and guide treatment decisions. The isolated subepithelial pattern may suggest the necessity for step-up therapy, closer monitoring, or additional testing, such as urea breath tests or stool *H. pylori* antigen tests, in accordance with current guidelines (24). Using IHC, *H. pylori* density grading can be easily summarized on the basis of the groups described in the methodology for clinical implications.

The overall pattern should be reported under the heading '*H. pylori*-positive cases' followed by a description of the specific location. This approach would improve communication with clinicians, for example, 'isolated surface epithelial pattern (surface bacteria 3, negative subepithelial signal)' or 'non-specific pattern (surface bacteria 3, subepithelial signal 2)'. This pattern group system aims to improve description of *H. pylori* behavior and could guide further management, particularly for the isolated subepithelial pattern group. In such cases, patients should undergo a urea breath test or a stool *H. pylori* antigen test, according to guidelines. The presence of this pattern suggests that step-up therapy and close follow-up or further investigation should be considered. By contrast, the surface-attached groups had a greater likelihood of successful treatment than did the isolated subepithelial pattern group.

Although the present study provides valuable insights into *H. pylori*, it has several limitations. The primary limitations are its retrospective design and the small number of patients with complete clinical follow-up data, which may affect the generalizability of the findings. Additionally, since only omeprazole is included in the national reimbursement program, other PPIs could have varying effects on the outcomes. Further prospective studies with larger cohorts and broader medication coverage are needed to validate and expand upon these findings.

In conclusion, *H. pylori*, particularly in subepithelial locations, can be underdiagnosed even with IHC tests. The detection rate is influenced by the sensitivity of the primary antibody used and the location of the bacteria. The effect of PPIs might prevent surface bacterial translocation. This feature should be included in pathological assessments and reports as an ancillary study alongside the updated Sydney System. Subepithelial *H. pylori* infection could serve as an independent prognostic factor for clinical outcomes and could guide further patient management.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

KL and CS conceived and designed the study, conducted the literature review, analyzed and interpreted the data. CS wrote the manuscript. KL revised the manuscript and performed the statistical analysis. CS and KL confirm the authenticity of all the raw data. Both authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The study protocol was approved by the ethics committee of the Navamindrajhiraj University (approval no. 120/2566; Bangkok, Thailand) before study initiation. The Ethics Committee/Institutional Review Board waived the requirement of written informed consent for participation from the participants or the participants' legal guardians/next of kin because due to the retrospective nature of the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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