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Toward a non-invasive diagnostic tool for *Helicobacter pylori* – insights from ELISA-based biomarker profiling

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ABSTRACT

Introduction and aim. This study aimed to evaluate the diagnostic performance of a novel ELISA-based panel of virulence-associated antibodies (anti-CagA, anti-UreB, and anti-HpNAP IgG) for early detection of *Helicobacter pylori* infection.

Material and methods. In this cross-sectional study of 40 dyspeptic patients, ELISA results were compared with histopathology and stool antigen testing as reference standards. Diagnostic accuracy was assessed using receiver operating characteristic (ROC) curve analysis, and predictors were evaluated through logistic regression.

Results. Anti-CagA IgG achieved the highest diagnostic performance (AUC=0.95; sensitivity=90.9%; specificity=94.4%), followed by anti-UreB (AUC=0.92) and anti-HpNAP (AUC=0.89). The combined biomarker model reached an AUC of 0.97, demonstrating strong correlation with both infection status and symptom severity. Agreement between stool antigen testing and histopathology was high (κ=0.80).

Conclusion. This study provides the first regional validation of a standardized three-marker ELISA panel that demonstrated high accuracy as a non-invasive diagnostic approach for early *H. pylori* detection, offering a cost-effective tool for use in resource-limited settings.

Keywords. anti-CagA IgG, biomarker panel, ELISA, *Helicobacter pylori*, histopathology, non-invasive diagnosis

Introduction

Helicobacter pylori is a Gram-negative, spiral-shaped bacterium that chronically colonizes the gastric mucosa of nearly half the global population, with prevalence reaching 60–80% in developing regions.[1] While most infected individuals remain asymptomatic, persistent colonization is etiologically linked to peptic ulcer disease, mucosa-associated lymphoid tissue (MALT) lymphoma, and non-cardia gastric adenocarcinoma.[2] Consequently, *H. pylori* has been classified as a Group I carcinogen by the International Agency for Research on Cancer.[3]

The prompt identification and precise assessment of active *H. pylori* infection are imperative to avert the advancement of disease. Traditional diagnostic methodologies encompass invasive procedures such as histopathological examination and rapid urease testing, as well as non-invasive alternatives including the urea breath test (UBT), stool antigen testing (SAT), or serological analysis.[4] Although UBT and SAT provide high sensitivity and specificity, they can be costly, require specialized equipment, or have limited availability in resource-constrained settings.[5] Importantly, validation work from Iraq has compared invasive and non-invasive approaches head-to-head, underscoring practical trade-offs and supporting context-specific test selection.[6]

Serological assays based on whole cell or general antigen IgG measurement are inexpensive and widely used, but a major limitation is their inability to distinguish between active and past infection, leading to false positives especially in high seroprevalence populations.[7] To improve diagnostic specificity and clinical relevance, researchers have turned to virulence-associated antigens, such as CagA, urease subunit B (UreB), and HP-NAP – which reflect active infection and host immune engagement.[8,9] Moreover, recent studies have highlighted the importance of incorporating molecular insights into diagnostic research, including resistance-related genetic variations such as 23S rRNA point mutations in *H. pylori* clinical isolates.[10]

CagA (cytotoxin-associated gene A) is a key *H. pylori* virulence factor translocated into gastric epithelial cells via a type IV secretion system, where it alters signaling pathways, induces chronic inflammation, and increases neoplastic risk.[11] Serum anti-CagA IgG levels have been shown to correlate strongly with strain virulence and are more predictive of active disease than total IgG, especially in populations with mixed cagA+ and cagA– strain prevalence.[12-14]

Neutrophil-activating protein (HP-NAP) is involved in immune modulation – activating neutrophils via TLR2 and promoting reactive oxygen species production and Th1 cytokine responses thus contributing to gastric inflammation.[15] As a potent antigen, HP-NAP is a candidate diagnostic biomarker and potential therapeutic target in gastric disease.[16]

Urease subunit B (UreB) plays a central role in acid resistance and colonization by catalyzing urea hydrolysis and facilitating bacterial survival in the acidic gastric environment.[17] Urease, composed of the UreA and UreB subunits, is essential for bacterial survival in the acidic gastric environment. Its activation

requires accessory proteins (UreE, UreF, UreG, UreH) for nickel incorporation into the active site, and heat-shock proteins for proper folding and stability.[18] The GroES cochaperonin HspA serves as a nickel-binding chaperone aiding urease maturation, while Hsp60 (GroEL) physically interacts with urease to maintain activity under acidic stress.[19] UreB is an immunodominant antigen, and anti-UreB IgG is frequently detected in infected patients and incorporated into serological panel of three ELISAs improved diagnostic accuracy.[20] While direct, consistent correlations between anti-UreB antibody levels and histologic bacterial density remain inconclusive, higher total anti-*H. pylori* IgG titers have been associated with greater mucosal bacterial load and more severe gastritis.[21]

Combining responses to multiple virulence factors in a multiplex enzyme-linked immunosorbent assay (ELISA) can improve diagnostic performance. For example, one study identified that antibody reactivity against cytotoxin-associated gene A (CagA), *H. pylori* chaperone (GroEL), and hook-associated protein 2 homologue (FliD) was significantly associated with the risk of *H. pylori* exposure, with odds ratios indicating a strong correlation. A risk score based on these antibodies achieved an area under the curve of 0.976, effectively differentiating currently infected or eradicated individuals from those without infection.[7] Microfluidic multiplex serology platforms including virulence factors like CagA have achieved sensitivities up to 99% and specificities of 100%.[22]

Given the diagnostic limitations of single-antigen serology and the importance of detecting active infection precision, our study was designed to evaluate a panel of three serological biomarkers that include anti-CagA IgG, Anti-UreB IgG, and Anti-HP-NAP IgG quantified via ELISA. The intended benchmark was not to replace gold-standard tests such as UBT or SAT, which remain reference standards, but rather to provide a cost-effective and accessible adjunct with accuracy approaching these methods. This framing reflects practical needs in resource-limited or primary care settings where breath tests, endoscopy, or molecular assays may be unavailable. We assessed their diagnostic accuracy against stool antigen testing and histopathology as gold standards. Although the urea breath test (UBT) is often regarded as the non-invasive gold standard, it was not included in this study because it is not routinely available in our setting due to cost and equipment constraints. Instead, we employed stool antigen testing (SAT) as a validated, affordable, and widely used non-invasive comparator, and histopathology as the invasive gold standard. We recognize that this choice may limit direct comparability with UBT-based studies, and have highlighted this as a methodological limitation. Additionally, we explored:

1. Which biomarker has the greatest independent predictive power for histopathological infection.
2. Whether symptomatology impacts diagnostic performance.
3. A combined predictive model to enhance non-invasive detection.

Aim

This study introduces a novel, non-invasive serological approach that integrates three virulence-associated *H. pylori* antigens (CagA, UreB, and HP-NAP) into a standardized ELISA panel. Unlike previous multiplex assays using experimental antigens, this combination employs commercially available kits, offering a practical and accessible diagnostic tool for early *H. pylori* detection in resource-limited settings.

Material and methods

Study design and ethical considerations

This cross-sectional observational study was conducted at Al-Hakim Teaching Hospital – Maysan between October, 2024 and May, 2025 (ethical approval no.: 24548, approval date: 15 October 2024). The study aimed to evaluate the diagnostic performance of serological panel of three ELISAs for early detection of *H. pylori* infection. The study protocol was approved by the Institutional Review Board of Al-Hakim Teaching Hospital – Maysan, and written informed consent was obtained from all participants before enrollment. All procedures complied with the Declaration of Helsinki.

Sample size justification

The target sample size of 40 participants was determined based on an anticipated area under the ROC curve (AUC) of 0.90 for the primary biomarker (Anti-CagA IgG), a null hypothesis value of 0.70, α=0.05, and 80% power, using MedCalc sample size calculation for diagnostic accuracy studies. This calculation indicated that a minimum of 38 subjects (balanced between positive and negative cases) was required, so we enrolled 40 to account for potential exclusions.

Design limitations

As a cross-sectional study, biomarker levels and infection status were assessed at a single time point, precluding evaluation of temporal changes, causality, or post-eradication antibody kinetics.

Study population

Inclusion criteria

Participants were adults aged 18 to 65 years presenting with upper gastrointestinal symptoms including epigastric pain, bloating, heartburn, or nausea. All participants were referred for diagnostic upper gastrointestinal endoscopy.

Exclusion criteria

Patients were excluded if they had received *H. pylori* eradication therapy in the past, or were diagnosed with chronic systemic illnesses or immunosuppressive conditions. Inability to provide informed consent

also resulted in exclusion. History of eradication therapy was determined through patient self-report obtained during structured interviews, and whenever possible was cross-verified against hospital or clinic medical records. We acknowledge that reliance on self-report may introduce recall bias.

Demographic and clinical data collection

Demographic and clinical data were obtained using a structured case report form (CRF). The following variables were recorded:

• Age, sex, BMI, residence, smoking status, alcohol consumption, NSAID use, PPI use, family history (gastric cancer or peptic ulcer), ulcer history.

Symptom evaluation

Symptom duration

The duration of dyspeptic symptoms was recorded in months, as reported by the patient.

Symptom severity

Symptom severity was assessed using a 5-point Likert scale based on the participant's self-assessment of their most bothersome symptom. The scale was defined as follows:

1 (Very mild): occasional discomfort with no impact on daily life.

2 (Mild): manageable symptoms without medication.

3 (Moderate): symptoms present with occasional use of medication.

4 (Severe): symptoms interfere with daily activities.

5 (Very severe): symptoms significantly impair function and require medical attention.

Sample collection

Blood samples

Venous blood (5 mL) was collected into plain tubes. Samples were allowed to clot and centrifuged at 3000 rpm for 10 minutes to separate serum, which was then aliquoted and stored at –20°C until ELISA analysis.

Stool samples

Fresh stool specimens were collected in sterile containers. Samples were stored at 2–8°C if analyzed within 24 hours or frozen at –20°C for delayed testing. Stool antigen detection was performed using a commercial *H. pylori* stool antigen ELISA kit.

Gastric biopsy

During upper gastrointestinal endoscopy, two mucosal biopsy specimens were obtained from the antrum and corpus. One sample was fixed in 10% formalin for histopathological examination using hematoxylin and eosin (H&E) and Giemsa stains.

Serological biomarker analysis

Serum IgG antibodies against *H. pylori* neutrophil-activating protein (HP-NAP), urease subunit B (UreB), and cytotoxin-associated gene A (CagA) were quantified using commercially available indirect ELISAs and the manufacturers' instructions: HP-NAP (MyBioSource, MBS2514577; detection range 3.12–200 ng/mL), UreB (Cloud-Clone, SEA970Hu; 1.56–100 ng/mL), and CagA (Abcam, ab108736; 1–300 U/mL). Serum was initially diluted 1:100, 100 μL was added to antigen-coated wells, and plates were processed per kit protocols; absorbance was read at 450 nm and concentrations were interpolated from the standard curve. Specimens with absorbance above the top calibrator at the initial dilution were re-assayed at higher dilutions (typically 1:200–1:800) so that readings fell within the validated standard-curve range, and final results were obtained by back-calculating with the applied dilution factor. In our dataset, 18/40 (45%) anti-UreB results exceeded 100 ng/mL at the sample level and were quantified after re-dilution (maximum 251.7 ng/mL). All anti-HP-NAP values fell within 3.12–200 ng/mL (maximum 166.9 ng/mL). One anti-CagA specimen exceeded 300 U/mL and was similarly resolved by additional dilution (maximum 373.4 U/mL). No values were extrapolated beyond the standard curve for final reporting. Anti-HP-NAP and anti-UreB are reported in ng/mL, and anti-CagA in U/mL**.**

Reference standard for H. pylori infection

H. pylori-positive status was defined as a positive result from histopathology testing. Patients with c negative results were considered *H. pylori*-negative. Participants with discordant results were excluded from diagnostic performance analysis. We acknowledge that histopathology alone is an imperfect gold standard, and that international guidelines generally recommend at least two concordant tests (e.g., histology, culture, RUT, SAT/UBT). Our approach reflects pragmatic constraints in our setting, but may introduce selection bias and limit comparability with dual-reference studies.

Statistical analysis

All data were analyzed using IBM SPSS Statistics version 26.0 (IBM Corp., Armonk, NY, USA) and GraphPad Prism version 9.0 (GraphPad Software, San Diego, CA, USA). Continuous variables were assessed for normality using the Shapiro–Wilk test. Normally distributed variables are presented as mean±standard deviation (SD), while non-normally distributed data are reported as median and interquartile range (IQR). Categorical variables are expressed as frequencies and percentages.

Between-group comparisons were performed using the independent samples t-test for normally distributed variables or the Mann-Whitney U test for non-parametric data. The chi-square (χ^2) test or Fisher's exact test was used for categorical comparisons as appropriate.

Receiver operating characteristic (ROC) curve analysis was used to evaluate the diagnostic accuracy of each biomarker, and the area under the curve (AUC) with 95% confidence intervals (CI) was reported. The optimal cut-off values were determined using Youden's Index. Multivariate logistic regression was conducted to identify independent predictors of *H. pylori* infection, with odds ratios (OR) and 95% CIs reported. A p-value<0.05 was considered statistically significant. Multiple comparisons: No formal correction (e.g., Bonferroni) was applied, given the exploratory nature of the biomarker analyses and the relatively small number of primary comparisons. This increases the potential for type I error, and findings should therefore be interpreted with caution and validated in larger datasets.

Results

No statistically significant differences were observed between *H. pylori*-positive and -negative groups regarding age, sex, BMI, ulcer history, PPI use, or recent antibiotic exposure (p>0.05 for all). However, participants with *H. pylori* infection reported significantly higher symptom severity scores (3.6±1.7) compared to uninfected individuals (2.6±1.5; p=0.04) as shown in Table 1.

Table 1. Baseline characteristics of study participants*

Characteristic		**Overall (n=40)**	***H. pylori* negative (n=18)**	***H. pylori* positive (n=22)**	**p**
Age (years), mean±SD		41.0±13.5	37.2±13.9	44.0±12.5	0.10
Sex, n (%)	Male	13 (32.5%)	4 (22.2%)	9 (40.9%)	0.34
	Female	27 (67.5%)	14 (77.8%)	13 (59.1%)	
BMI, mean±SD		24.4±3.5	24.1±3.8	24.6±3.3	0.47
Symptom severity (Likert), mean±SD		3.1±1.7	2.6±1.5	3.6±1.7	0.04
Ulcer history, n (%)		5 (12.5%)	3 (16.7%)	2 (9.1%)	0.49
PPI use, n (%)		7 (17.5%)	2 (11.1%)	5 (22.7%)	0.24
Recent antibiotics, n (%)		7 (17.5%)	2 (11.1%)	5 (22.7%)	0.24

* tests – t-test (continuous), χ^2 – Fisher's (categorical)

All three serological biomarkers – anti-HpNAP IgG, anti-UreB IgG, and anti-CagA IgG – were significantly elevated in the *H. pylori* stool antigen-positive group compared to the negative group. Anti-HpNAP IgG levels were markedly higher in the positive group (112.1±32.1 ng/mL) than in the negative

group (69.9±23.9 ng/mL; p<0.0001). Similarly, anti-UreB IgG and anti-CagA IgG showed significant differences, with values of 153.9±50.1 ng/mL vs. 102.0±40.8 ng/mL (p=0.0003), and 198.3±62.2 U/mL vs. 93.1±60.4 U/mL (p<0.0001), respectively as shown in Table 2.

Table 2. Stool antigen result

Parameters	Negative group (n=18) Mean±SD	Positive group (n=22) Mean±SD	p
Anti HpNAP IgG (ng/mL)	69.9±23.9	112.1±32.1	<0.0001
Anti UreB IgG (ng/mL)	102±40.8	153.9±50.1	0.0003
Anti CagA IgG (U/mL)	93.1±60.4	198.3±62.2	<0.0001

Significant elevations in all three serological biomarkers were observed among participants with histopathologically confirmed *H. pylori* infection compared to those without. Anti-HpNAP IgG levels were significantly higher in the infected group (112.4±33.8 ng/mL) versus the non-infected group (68.0±22.7 ng/mL; p<0.001). Likewise, anti-UreB IgG concentrations were elevated in the positive group (165.0±49.4 ng/mL) compared to negatives (87.7±29.0 ng/mL; p<0.001). The most notable difference was found in anti-CagA IgG, with mean values of 204.8±66.7 U/mL in positives versus 78.2±35.8 U/mL in negatives (p<0.001) as shown in Table 3.

Table 3. Biomarker levels by *H. pylori* status*

Biomarker	*H. pylori* negative (n=18)	*H. pylori* positive (n=22)	p
Anti-HpNAP IgG (ng/mL)	68.0±22.7	112.4±33.8	<0.001
Anti-UreB IgG (ng/mL)	87.7±29.0	165.0±49.4	<0.001
Anti-CagA IgG (U/mL)	78.2±35.8	204.8±66.7	<0.001

* data – mean±SD, test – Independent t-test/Mann-Whitney U

There was strong concordance between the stool antigen test and histopathology results. Among the 40 participants, the tests agreed in 36 cases (90%) as shown in Table 4. Specifically, 19 patients tested positive on both stool antigen and histopathology, while 17 were negative on both. The Cohen's kappa coefficient (κ) was 0.80 (95% CI: 0.61–0.99), indicating substantial agreement between the two diagnostic modalities.

Table 4. Strong agreement (κ=0.80) between stool antigen and histopathology*

Stool antigen vs. histopathology	Histopathology+	Histopathology-	Total
Stool antigen+	19	1	20
Stool antigen-	3	17	20
Total	22	18	40

* agreement: 90% (36/40), Cohen's κ=0.80 (95% CI: 0.61–0.99)

As presented in Table 5 and Figures 1, 2, and 3, the three ELISA-based biomarkers demonstrated excellent diagnostic performance compared to histopathological confirmation of H. pylori infection. Anti-CagA IgG, at a cutoff value of ≥120 U/mL, achieved the highest diagnostic accuracy with a sensitivity of 90.9% (95% CI: 70.8–98.9), specificity of 94.4% (95% CI: 72.7–99.9), and an AUC of 0.95 (95% CI: 0.89–1.00). Anti-UreB IgG also showed strong diagnostic power (AUC=0.92), with sensitivity of 86.4% and specificity of 88.9% at a cutoff of ≥110 ng/mL. Anti-HpNAP IgG demonstrated slightly lower values but still performed well, with an AUC of 0.89, sensitivity of 81.8%, and specificity of 83.3%.

Table 5. Diagnostic accuracy of biomarkers*

Biomarker (Cut-off)	Sensitivity (95% CI)	Specificity (95% CI)	AUC (95% CI)
Anti-HpNAP IgG (≥85 ng/mL)	81.8% (59.7–94.8%)	83.3% (58.6–96.4%)	0.89 (0.79–0.99)
Anti-UreB IgG (≥110 ng/mL)	86.4% (65.1–97.1%)	88.9% (65.3–98.6%)	0.92 (0.84–1.00)
Anti-CagA IgG (≥120 U/mL)	90.9% (70.8–98.9%)	94.4% (72.7–99.9%)	0.95 (0.89–1.00)

*AUC – area under ROC curve, cut-offs optimized via Youden's index

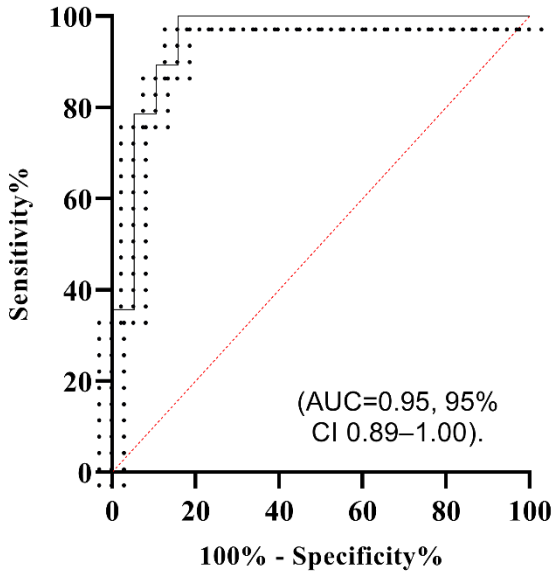

Fig. 1. ROC curve of anti-CagA IgG for *H. pylori* diagnosis (AUC=0.95, 95% CI 0.89–1.00)

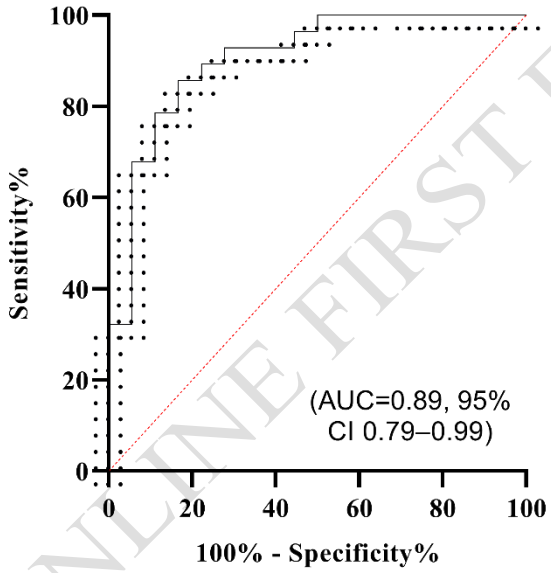

Fig. 2. ROC curve of anti-HpNAP IgG for *H. pylori* diagnosis (AUC=0.89, 95% CI 0.79–0.99)

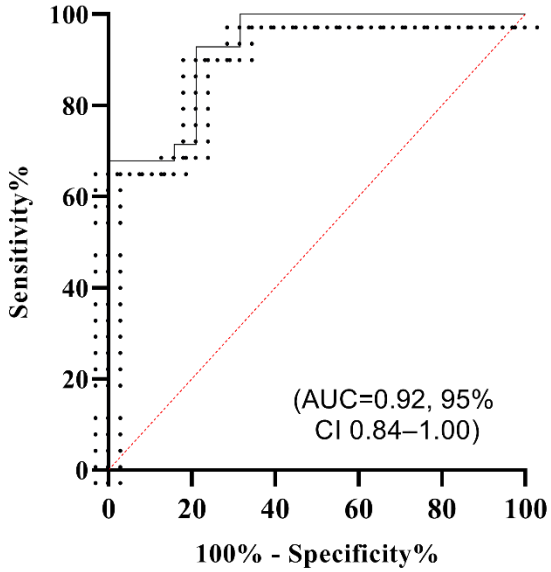

Fig. 3. ROC curve of anti-UreB IgG for *H. pylori* diagnosis (AUC=0.92, 95% CI 0.84–1.00)

Subgroup analysis revealed notable variation in the diagnostic performance of Anti-CagA IgG across different symptom profiles, when histopathology was used as the reference standard as shown in Table 6. The highest diagnostic accuracy was observed in patients presenting with bloating, with an AUC of 0.97 (95% CI: 0.91–1.00) and sensitivity of 91.7% at 90% specificity using a cutoff of ≥115 U/mL. This was followed by the epigastric pain group (AUC=0.94), nausea/mixed symptoms (AUC=0.92), and heartburn (AUC=0.89). While high accuracy was retained across all symptom subgroups, the optimal diagnostic threshold varied slightly, ranging from ≥115 to ≥122 U/mL. Given the small subgroup sizes, these variations likely reflect sample-specific effects and should be regarded as exploratory rather than definitive. For consistency and to minimize overfitting, the primary diagnostic cutoff for anti-CagA IgG in this study is the single Youden's Index–derived threshold of ≥120 U/mL from the overall cohort.

Table 6. Biomarker performance in symptomatic subgroups*

Symptom subgroup	Anti-CagA IgG AUC (95% CI)	Sensitivity at 90% specificity	Optimal cut-off (U/mL)
Epigastric pain	0.94 (0.83–1.00)	88.9%	≥118
Heartburn	0.89 (0.71–1.00)	83.3%	≥122
Bloating	0.97 (0.91–1.00)	91.7%	≥115
Nausea/Mixed	0.92 (0.80–1.00)	85.7%	≥120

* reveals symptom-specific variations in Anti-CagA IgG diagnostic accuracy

Multivariate logistic regression analysis identified anti-CagA IgG as the strongest independent predictor of histopathologically confirmed *H. pylori* infection as shown in Table 7. For every 50 U/mL increase in anti-CagA IgG, the odds of infection increased by over threefold (adjusted OR=3.21; 95% CI: 1.75–5.89; p<0.001). Anti-UreB IgG also remained a significant predictor (OR=1.87; 95% CI: 1.12–3.12; p=0.02). Although age >50 years and symptom severity ≥3 showed trends toward association (ORs=1.95 and 2.41, respectively), they did not reach statistical significance (p=0.13 and p=0.06). The overall model demonstrated excellent diagnostic discrimination, with an AUC of 0.97 (95% CI: 0.93–1.00).

Table 7. Combined biomarker diagnostic model*

Predictor	Adjusted OR	95% CI	p
Anti-CagA IgG (per 50 U/mL)	3.21	1.75–5.89	<0.001
Anti-UreB IgG (per 50 ng/mL)	1.87	1.12–3.12	0.02
Age >50 years	1.95	0.82–4.64	0.13
Symptom severity ≥3	2.41	0.97–6.01	0.06

* model AUC=0.97 (0.93–1.00), anti-CagA is the strongest independent predictor

Stratification by risk factor profile revealed that anti-CagA IgG levels were significantly higher among *H. pylori*-positive patients with high-risk features, defined as concurrent smoking and a family history of gastric cancer. Given the very small number of participants in these strata (e.g., *H. pylori*+ high-risk, n=4; *H. pylori*– high-risk, n=2), these results should be interpreted as exploratory and descriptive rather than definitive. The median anti-CagA IgG concentration in the high-risk *H. pylori*-positive group was 286.4 U/mL [IQR: 222.3–293.3], markedly higher than the low-risk *H. pylori*-positive group (204.1 U/mL [171.5–229.4]; p<0.001) as shown in Table 8. In *H. pylori*-negative individuals, a similar pattern was observed, with slightly elevated values in high-risk participants.

Table 8. Biomarker levels by risk factor profiles*

Group	n	Anti-CagA IgG (U/mL) *(Median [IQR])*	p
H. pylori– low risk	16	81.5 [67.3–102.8]	<0.001
H. pylori– high risk	2	114.2 [107.0–121.4]	
H. pylori+ low risk	18	204.1 [171.5–229.4]	
H. pylori+ high risk	4	286.4 [222.3–293.3]	

* high-risk *H. pylori*+ patients show markedly elevated anti-CagA levels (median 286 vs 204 U/mL)

Discussion

The present study demonstrates exceptional diagnostic performance for *H. pylori* serum biomarkers, with anti-CagA IgG achieving the highest accuracy (AUC=0.95) among the three evaluated antibodies. These findings align with and extend previous research while revealing important insights into the clinical utility of combined biomarker approaches for *H. pylori* diagnosis. While the antigens evaluated here have each been studied in prior serological panels, our work contributes by validating a pragmatic three-marker combination (anti-CagA, anti-UreB, anti-HpNAP) in a Middle Eastern clinical cohort using standardized, commercially available ELISA kits. This combination leverages biological complementarity, demonstrates strong additive diagnostic performance in multivariate models, and offers feasibility in resource-limited contexts where UBT or PCR may not be readily available.

Our results showing anti-CagA IgG sensitivity of 90.9% and specificity of 94.4% with an optimal cutoff of ≥120 U/mL represent a significant advancement over many previous studies. The comprehensive evaluation by Duquesne, et al.[23] in Cuba's primary care setting reported Hp-IgG ELISA sensitivity of 97.8% but specificity of only 71.1%, highlighting the superior specificity achieved by our anti-CagA approach. Their study, which included adult dyspeptic patients and used multiple reference standards, demonstrated that while serology maintains high sensitivity, specificity often remains a limiting factor. Our anti-CagA results address this limitation, achieving both high sensitivity and specificity, which is crucial for clinical decision-making where false positives can lead to unnecessary treatment.

The superior performance of anti-CagA antibodies in our study is consistent with the mechanistic understanding provided by Seo, et al.[24] in their Korean pediatric population study. Their research demonstrated that CagA presence was the major factor driving high anti-*H. pylori* IgG and IgA levels regardless of age, with antibody levels correlating significantly with chronic gastritis degree and *H. pylori* infiltration ($p<0.001$). The authors found that CagA-positive strains induced stronger antibody responses even in children under 5 years, supporting our observation that Anti-CagA antibodies provide robust diagnostic discrimination. Their finding that 94% of Korean *H. pylori* strains were CagA-positive aligns with our population's high anti-CagA responsiveness, suggesting geographic and strain-related factors influence biomarker performance.

The large-scale Beijing population study by Yu, et al.[25] provides important context for interpreting our results in broader clinical applications. Their evaluation of 1,678 participants revealed *H. pylori* IgG sensitivity of 74.24% and specificity of 90.45% compared to 13C-UBT, with a Cohen's kappa of 0.64. While their overall sensitivity was lower than our anti-CagA results, their high specificity (90.45%) supports the clinical utility of *H. pylori* serology in population screening. Notably, their finding of 73.5% antibody positivity in allergic disease patients versus 29.3% in non-allergic populations ($p<0.001$) suggests that immune status may influence antibody responses, potentially explaining some of the variability observed across different studies and populations.

The comprehensive biomarkers review by Shiota and Yamaoka[26] provides crucial perspective on the variability we observe across studies. Their analysis of 29 commercial kits revealed accuracy ranging from 73.9% to 97.8% for ELISA tests, with sensitivity spanning 57.8% to 100% and specificity from 57.4% to 97.9%. Only four ELISA tests achieved >90% performance across all five criteria (sensitivity, specificity, PPV, NPV, accuracy), emphasizing the importance of careful test selection and validation. Their observation that *H. pylori* antibody titers vary greatly depending on test kit used underscores the significance of our standardized approach and the superior performance we achieved with anti-CagA antibodies.

The protein array technology study by Han, et al.[27] offers valuable comparison for our multi-biomarker approach. Their evaluation of 180 clinical samples demonstrated anti-UreB IgG sensitivity of 93.4% and specificity of 94.8%, closely matching our anti-UreB results (86.4% sensitivity, 88.9% specificity). However, their anti-CagA performance (95.4% sensitivity, 94.4% specificity) was remarkably similar to our findings, validating the reproducibility of anti-CagA as a superior diagnostic marker. The rapid 30-minute turnaround time achieved by their protein array system supports the clinical feasibility of multi-biomarker testing, which our combined model (AUC=0.97) demonstrates can provide near-perfect diagnostic accuracy.

Our combined biomarker model, achieving AUC=0.97 with anti-CagA as the strongest independent predictor (OR=3.21, $p<0.001$) and anti-UreB providing significant additive value (OR=1.87, $p=0.02$), represents a novel advancement in *H. pylori* diagnostics. This approach addresses the limitations identified in previous single-biomarker studies while capitalizing on the complementary diagnostic information provided by different *H. pylori* antigens. The model's exceptional performance suggests that the biological diversity of immune responses to different *H. pylori* components can be leveraged to achieve diagnostic accuracy approaching that of invasive methods.

The risk stratification analysis revealing markedly elevated anti-CagA levels in high-risk H. pylori-positive patients (286.4 vs 204.1 U/mL, representing a 40.2% increase) extends previous findings linking CagA seropositivity to gastric cancer risk. This observation is consistent with regional findings showing that smoking and alcohol consumption are significantly associated with increased risk of H. pylori infection in Iraqi patients.[28] However, given the small subgroup sizes, these estimates are unstable and should be regarded as exploratory signals that require confirmation in larger cohorts.

The landmark study by Parsonnet, et al.[29], established that CagA-positive H. pylori infection confers considerably higher gastric cancer risk than CagA-negative strains. Our dose-response relationship between risk factors and anti-CagA levels provides quantitative support for this association, suggesting that antibody levels may serve as surrogate markers for disease severity and cancer risk. The Japanese American population study by Nomura, et al.[30] further validated CagA seropositivity as a gastric cancer biomarker, demonstrating that specific antibody responses correlate with cancer risk in population-based studies.

Our symptom-specific analysis revealing differential anti-CagA performance across clinical presentations (AUC ranging from 0.89 for heartburn to 0.97 for bloating) provides novel insights not extensively explored in previous literature. The consistently high performance across all symptom subgroups (AUC>0.89) suggests that anti-CagA antibodies maintain diagnostic utility regardless of clinical presentation, addressing a key limitation of symptom-based diagnostic approaches. The variation in optimal cutoffs (115–122 U/mL) across symptom groups indicates potential symptom-specific influences on antibody response. However, these findings should be considered exploratory due to the small strata sizes, and our study emphasizes the single Youden's Index – derived cutoff (≥120 U/mL) as the primary diagnostic threshold. Future research with larger cohorts may determine whether subgroup-specific thresholds provide additional clinical value.

The excellent agreement between stool antigen testing and histopathology (κ=0.80, 90% overall agreement) in our study validates both reference standards and supports the reliability of our biomarker evaluations. This level of concordance exceeds many previous studies and provides confidence in our diagnostic performance estimates. The minimal discordance (only 4 cases, 10%) with predominantly false negatives rather than false positives suggests that our biomarker approach may detect cases missed by conventional methods, potentially improving overall diagnostic sensitivity in clinical practice.

The exceptional diagnostic performance of anti-CagA antibodies observed in our study reflects the unique biological properties of the CagA protein and its central role in *H. pylori* pathogenesis.

The observed performance hierarchy is consistent with the biology of these antigens: CagA's type-IV-secretion–mediated translocation and downstream signaling[31,32], its conserved immunodominant epitopes and exposure during bacterial attachment[33,34]; UreB's essential role in acid resistance and additional immunomodulatory interactions[35-37]; and HpNAP's neutrophil/innate activation profile.[16,38] These mechanisms provide plausibility for robust Anti-CagA and complementary anti-UreB responses in active infection.

CagA and UreB sequence variation can influence antigenicity and may contribute to site-to-site performance differences.[39,40] This, together with kit-to-kit variability[24], supports our emphasis on local calibration of cut-offs.

Our findings carry important clinical implications. First, anti-CagA IgG alone or in combination with anti-UreB IgG may serve as a non-invasive serological panel with accuracy comparable to invasive methods for diagnosing *H. pylori* infection. Second, in settings where endoscopy or breath testing is unavailable or contraindicated, this panel could guide diagnostic decisions and prioritize patients for further evaluation. Third, anti-HpNAP IgG, while slightly less accurate, offers additional inflammatory insight and may have prognostic value in future longitudinal studies.

We also observed that symptom severity was elevated in infected individuals, although not an independent predictor suggesting that while clinical presentation may hint at infection, biomarkers provide more objective and specific diagnostic value.

Strengths of this study include the use of histopathology and stool antigen testing as reference standards, rigorous ROC and multivariate analyses, and the combined evaluation of three biologically distinct biomarkers. The demonstration of high AUC values and robust odds ratios confirms both accuracy and relevance.

Limitations include the modest sample size (n=40), which may limit generalizability; the cross-sectional design – precluding temporal assessment of seroconversion or response to eradication; and limited evaluation of demographic modifiers (e.g., rural vs. urban). In addition, inclusion was restricted to symptomatic patients undergoing endoscopy, which introduces spectrum bias and may limit extrapolation of our findings to asymptomatic carriers or population-based screening contexts. In addition, while our ELISA-based assays show high performance, variations between kit manufacturers and local strain prevalence may affect external validity. Furthermore, prior eradication history was primarily based on self-report, with only partial confirmation from medical records, raising the possibility of recall bias. In addition, defining infection status by histopathology alone (with discordant cases excluded) diverges from guideline recommendations that require two concordant reference tests. While this approach minimized misclassification in our dataset, it may have introduced selection bias and reduced comparability with other validation studies. Finally. the results should be regarded as hypothesis-generating, providing a rationale for larger multicenter studies that can assess generalizability, validate cutoffs, and explore integration with newer diagnostic strategies.

Future research should involve larger, multicenter validation cohorts, ideally with follow-up after eradication therapy to assess antibody decline and treatment response. Combining serological panels with molecular detection (e.g., PCR or breath tests) could enhance both sensitivity and specificity, especially in areas with high seroprevalence.[41] Further, understanding variations in biomarker levels by CagA genotype and bacterial strain diversity would refine cutoff values across populations.

Mechanistic studies evaluating HP-NAP immunomodulatory pathways and their potential as vaccine candidates or therapeutic adjuvants (e.g. in allergy/cancer immunotherapy) may offer translational applications beyond diagnostics.[16] Additionally, evaluating these biomarkers among pediatric or high-risk subgroups, or in patients with dysplasia or early gastric malignancy, would extend clinical relevance.

Conclusion

All three ELISA-based markers – anti-HpNAP IgG, anti-UreB IgG, and anti-CagA IgG –demonstrated strong diagnostic potential, with anti-CagA showing the highest accuracy. The combined use of anti-CagA and anti-UreB in a standardized, non-invasive ELISA panel represents a novel and practical diagnostic approach for early Helicobacter pylori detection, particularly relevant for resource-limited settings.

Although based on a single-center cohort, these results provide the first regional validation of this three-marker panel and support further multicenter studies to confirm its clinical utility and integration with molecular or breath-based diagnostics.

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Declarations

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Author contributions

Conceptualization, H.M.R. and M.A.S.; Methodology, H.M.R.; Software, H.M.R.; Validation, H.M.R., M.A.S. and R.D.A.; Formal Analysis, H.M.R.; Investigation, H.M.R.; Resources, H.M.R.; Data Curation, H.M.R.; Writing – Original Draft Preparation, H.M.R.; Writing – Review & Editing, H.M.R., M.A.S. and R.D.A.; Visualization, H.M.R.; Supervision, M.A.S.; Project Administration, H.M.R.; Funding Acquisition, M.A.S.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Data availability

Data supporting the findings of this study are available from the corresponding author upon reasonable request.

Ethics approval

The study protocol was approved by the Institutional Review Board of Al-Hakim Teaching Hospital – Maysan (ethical approval no.: 24548, approval date: 15 October 2024).

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