Journal of Physiology and Biology Sciences (JPBS)

E-ISSN: 3107-2283

journal homepage: https://physiobiojournal.com/

DOI: https://doi.org/10.69792/jpbs.2025.vol.2.2

Serum TNF-α, IL-8, IgA, and Stool Antigen as Immunological Markers of *Helicobacter pylori Infection: A Case-Control Immunoprofiling Study*

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Received: 18/10/2025
Revised: 11/12/2025
Accepted: 23/12/2022
Published: 31/12/2025

ABSTRACT

Background: Helicobacter pylori (H. pylori) infection orchestrates a coordinated innate–adaptive immune response involving TNF-α-driven mucosal amplification, IL-8-mediated neutrophilic recruitment, IgA humoral activation, and stool antigen reflecting direct bacterial load. Concurrent integrated immunoprofiling of these four mechanistically distinct axes within an Iraqi clinical cohort has not been previously reported in the literature.

Objectives: To characterise within-sample immunological differences between confirmed H. pylori cases and screened healthy controls across four biomarkers; to examine stool-antigen-stratum-dependent biomarker gradients; to evaluate the TNF-α/IL-8 ratio; and to identify the strongest confounder-adjusted independent predictor using Firth penalised logistic regression.

Methods: Two-gate case-control immunoprofiling study (University of Samarra, Iraq; March–September 2024): n=60 confirmed H. pylori cases (reference standard: ^{13}C-UBT + endoscopic biopsy/histopathology + RUT; stool antigen ELISA was measured as an index test and was not included in case definition) vs. n=30 screened healthy controls. Disease burden strata (mild/moderate/severe) were defined by the updated Sydney System histological grade, not by stool antigen tertiles. Statistical methods: Wilcoxon rank-sum; pROC DeLong AUC; Kruskal-Wallis + Dunn-Bonferroni; Spearman + Benjamini-Hochberg FDR; Firth penalised logistic regression (R package logistf v1.24, Heinze 2002). STARD 2015 reporting / QUADAS-2 bias-reduction framework applied.

Results: All four biomarkers showed substantially higher concentrations in cases than controls (rank-biserial r=1.00; FDR-corrected q<0.001 for all). Median concentrations (cases vs. controls): TNF-α 48.7 vs. 8.3 pg/mL; IL-8 186.4 vs. 32.1 pg/mL; IgA 3.84 vs. 1.21 mg/dL; stool antigen 102.5 vs. 14.2 U/mL. Within-sample AUC=1.000 for all biomarkers (upper-bound estimate under two-gate design; bootstrap-optimism-adjusted C=0.998). Stool antigen was the only biomarker with a statistically significant gradient across Sydney-graded severity strata (p<0.001). TNF-α/IL-8 ratio was stable across strata (p=0.612). Firth regression: TNF-α was the strongest independent predictor (adjusted OR=15.8; 95% CI 2.4–104.3; p=0.004).

Conclusion: Within this case-control immunoprofiling study, all four biomarkers show complete within-sample separation. Important methodological caveats apply: (1) the two-gate design with healthy controls inflates apparent discriminatory performance; (2) stool antigen was an index test and was excluded from case definition to avoid incorporation bias. These findings constitute Proof-of-Concept immunological data. Multi-centre prospective validation in consecutive symptomatic patient series, incorporating virulence genotyping, a validated clinical severity index, and formal incremental value analysis (NRI/IDI, DCA), is required before any clinical application.

KEYWORDS : *Helicobacter pylori; TNF-α; IL-8; IgA; Stool antigen; Immunoprofiling; Incorporation bias; STARD 2015.*

INTRODUCTION

Hobacter pylori (*H. pylori*), a Gram-negative microaerophilic spiral bacterium, chronically colonises the human gastric mucosa. A systematic review and meta-analysis estimated global prevalence at approximately 44.3% in 2015, with substantially higher rates in low- and middle-income countries, including Iraq and neighbouring Middle Eastern settings, where socioeconomic constraints, limited sanitation infrastructure, and overcrowded household conditions sustain high transmission rates.[1,2] H. pylori infection is formally classified as a Group I

definite carcinogen by the International Agency for Research on Cancer (IARC) and is causally linked to chronic active gastritis, peptic ulcer disease, gastric adenocarcinoma, and mucosa-associated lymphoid tissue (MALT) lymphoma.[3,4]

The immunopathogenesis of *H. pylori* infection engages both innate and adaptive immune axes. CagA protein, delivered into gastric epithelial cells via the type IV secretion system, activates NF-κB and MAPK pathways, driving interleukin-8 (IL-8/CXCL8) secretion and subsequent neutrophilic mucosal recruitment.[5,6] Tumour necrosis factor-alpha (TNF-α), produced by activated macrophages and gastric epithelial cells in response to *H. pylori* lipopolysaccharide and CagA signalling, amplifies this response through TNFR1-mediated epithelial apoptosis and systemic inflammatory amplification.[7,8] At the humoral level, persistent antigenic stimulation drives B-cell activation and IgA class switching in the gastric lamina propria, generating *H. pylori*-associated serum IgA as a marker of mucosal humoral memory.[9] Stool antigen ELISA directly quantifies intraluminal bacterial antigenic burden — mechanistically distinct from host immune activation markers and thus complementary rather than redundant to cytokine and antibody profiling.[10]

Despite extensive individual literatures on each of these biomarkers, their concurrent integrated evaluation — incorporating Firth-penalised logistic regression to address complete distributional separation, formal disease burden stratification using an independent histological severity index, and TNF-α/IL-8 ratio analysis — has not been previously reported in the Iraqi or broader regional clinical context. The present case-control immunoprofiling study was designed to address this gap. Study design and reporting follow STARD 2015 guidelines;[10] bias-reduction measures were applied in accordance with the four QUADAS-2 domains (participant selection, index test conduct, reference standard, and flow/timing).[11]

MATERIALS AND METHODS

2.1 Study Design and Ethical Approval

A two-gate case-control immunoprofiling study was conducted at the Clinical Immunology and Microbiology Laboratory, University of Samarra, Samarra, Saladin Governorate, Iraq (March–September 2024). Ethical approval: University of Samarra Institutional Review Board (No.: IRB-US-2024-011); conducted in accordance with the Declaration of Helsinki (2024 revision).[12] Written informed consent was obtained from all participants prior to enrolment. All records were pseudonymised at the point of collection. Anonymised data are available from the corresponding author upon reasonable request, subject to institutional ethical approval.

2.2 Sample Size

Sample size was calculated a priori using the Buderer (1996) method for diagnostic accuracy studies[13]: expected sensitivity/specificity 0.95, disease prevalence 0.50, acceptable absolute error w=0.10, two-sided α=0.05; minimum n=73 required. Enrolment of n=90 (60 cases + 30 controls) surpasses this threshold and supports estimation of AUC with a DeLong 95% CI width ≤0.08. Supplementary post-hoc power analysis (G*Power 3.1.9) was performed for completeness only and is not used as the primary justification.

2.3 Participant Selection

Cases (n=60): Adults aged ≥18 years with confirmed *H. pylori* infection, defined by concurrent positivity on BOTH of the following independent reference tests, which did NOT include any index biomarker under evaluation: (1) ^{13}C-urea breath test (DOB ≥4‰; FANci2, Fischer Analysen Instrumente, Germany); and (2) endoscopic gastric antrum biopsy with histopathological active gastritis graded using the updated Sydney System[26], with concurrent positive rapid urease test (CLOtest, Kimberly-Clark, USA). Stool antigen ELISA was collected as an index test for evaluation only and was NOT used to define infection status (QUADAS-2 Domain 3). Disease burden severity was stratified into three groups by updated Sydney System histological grade: mild (grade I, n=20), moderate (grade II, n=20), and severe (grade III, n=20), ensuring independence of the severity index from the stool antigen measurement. Exclusion criteria: prior *H. pylori* eradication within 12 months; autoimmune disease; immunosuppressive therapy; pregnancy; active malignancy; concurrent non-*H. pylori* gastrointestinal infection; proton pump inhibitor or antibiotic use within two weeks.

Controls (n=30): Adult volunteers from the same catchment population presenting with upper gastrointestinal symptoms but testing negative on both the ^{13}C-UBT and stool antigen ELISA (the latter used for screening only), with negative *H. pylori* serology, and no chronic inflammatory conditions, immunosuppressive therapy, or anti-

inflammatory/antibiotic medication within two weeks. Symptomatic controls were recruited to reduce spectrum bias relative to purely asymptomatic healthy volunteers (QUADAS-2 Domain 1).

2.4 Laboratory Assays

Venous blood (3–5 mL) was collected by trained phlebotomists; serum was separated by centrifugation (2,000×g, 10 min, 4°C), aliquoted (0.5 mL), and stored at −80°C with a maximum of two freeze-thaw cycles. Stool specimens were stored at −80°C within four hours of collection. All ELISA assays were performed in duplicate with calibration standards and external quality-control samples on each microplate; inter-plate batch effects were monitored using the QC values. Samples exceeding the assay's upper measurement range were diluted and re-assayed (STARD Items 6–8).[10]

Serum total IgA: Abcam Human IgA ELISA Kit (Cat. ab108848; UK); analyte: total serum IgA; LOD 0.16 mg/L; calibration range 0.156–10.0 mg/L; intra-assay CV 4.2%; inter-assay CV 7.8%. Results expressed in mg/dL after standard unit conversion.

Serum TNF-α: R&D Systems Quantikine Standard ELISA (Cat. STA00D; USA); LOD 0.106 pg/mL per manufacturer datasheet; calibration range 0–2,500 pg/mL; intra-assay CV 3.9%; inter-assay CV 8.1%. Samples with concentrations below LOD were assigned a value of LOD/√2 for analysis.

Serum IL-8: R&D Systems DuoSet ELISA (Cat. DY208; USA); LOD 3.5 pg/mL; calibration range 3.5–2,000 pg/mL; intra-assay CV 5.1%; inter-assay CV 9.3%. Sample dilution factor: 1:2 for all case specimens.

Stool antigen (index test only): RIDASCREEN *H. pylori* Stool Antigen ELISA (R-Biopharm AG, Germany); this is a qualitative kit for binary detection. For the purpose of this immunoprofiling study, stool antigen burden was quantified using the sample/cut-off (S/CO) absorbance ratio — a validated semi-quantitative approach reported in the literature for comparing antigen load across subjects[27]. Results are expressed as S/CO units. Stool antigen was measured as an index test independently of the reference standard by a separate laboratory operator (STARD Item 7).[10]

2.5 Statistical Analysis

Analyses were performed in R v4.3.0 and IBM SPSS v26 per SAMPL guidelines.[14] Normality was assessed by the Shapiro-Wilk test (all groups confirmed non-normal). Between-group comparisons used the Wilcoxon rank-sum test with rank-biserial r as the primary effect size (r=1.00 indicates complete distributional separation); 95% bias-corrected and accelerated (BCa) bootstrap confidence intervals (2,000 iterations). ROC analyses used the pROC package (Robin et al., 2011)[15] with DeLong 95% CIs; optimal thresholds by Youden index. Severity comparisons: Kruskal-Wallis + Dunn-Bonferroni post-hoc. Spearman correlations corrected for multiple comparisons using Benjamini-Hochberg FDR (controls the false discovery rate, not familywise error rate). Complete distributional separation between groups necessitated Firth penalised logistic regression (R package logistf version 1.24; Heinze & Schemper, 2002[16]) to obtain stable coefficient estimates; age and sex included as pre-specified covariates. The Hosmer-Lemeshow goodness-of-fit test was applied only as a supplementary calibration indicator and is interpreted with caution given the small sample size and complete separation context. Bootstrap internal validation (500 resamples) yielded the optimism-adjusted C-statistic. Statistical significance threshold: α=0.05 (two-sided) after FDR correction.

RESULTS

3.1 Participant Characteristics

Cases (n=60): mean age 38.5±11.2 years (35 male, 25 female). Controls (n=30): mean age 36.8±10.7 years (18 male, 12 female). Groups were comparable in age (Wilcoxon p=0.472) and sex (χ^2=0.831, p=0.362). Sydney System histological severity distribution: mild n=20, moderate n=20, severe n=20. Age and sex were non-significant covariates in the Firth logistic regression (both p>0.05). Table 5 presents baseline characteristics.

3.2 Between-Group Biomarker Comparisons

All four biomarkers demonstrated complete distributional separation between cases and controls (rank-biserial r=1.00; FDR-corrected q<0.001 for all comparisons), indicating that every infected participant had higher concentrations than every control participant. This result is interpreted with the explicit caveat that it constitutes an upper-bound within-sample estimate under the two-gate case-control design (see Discussion). Results are presented in Table 1.

Table 1. Biomarker concentrations and effect sizes: H. pylori cases vs. symptomatic controls.

Biomarker	Cases Median (IQR)	Controls Median (IQR)	p	r	Min. Gap	Cohen's d†
TNF-α (pg/mL)	48.7 (38.2–61.4)	8.3 (5.9–11.2)	<0.001	1.00	14.6	3.82
IL-8 (pg/mL)	186.4 (147.3–224.8)	32.1 (24.6–41.3)	<0.001	1.00	12.3	4.11
Total IgA (mg/dL)	3.84 (3.12–4.53)	1.21 (0.94–1.58)	<0.001	1.00	11.7	3.64
Stool Antigen (S/CO)	102.5 (72.4–131.8)	14.2 (9.8–19.6)	<0.001	1.00	10.8	4.47

Note. r = rank-biserial correlation (primary effect size; 1.00 = complete distributional separation). †Cohen's d provided as supplementary descriptor only; non-normality confirmed by Shapiro-Wilk in all groups. Min. Gap = minimum case value minus maximum control value. p-values are FDR-corrected (Benjamini-Hochberg). Stool antigen expressed as S/CO (sample/cut-off) absorbance ratio. All estimates are upper-bound within-sample values (see Section 4.1).

3.3 Within-Sample Diagnostic Accuracy

Within-sample AUC=1.000 (95% CI 1.000–1.000) for all four biomarkers (Table 2). As detailed in Section 4.1, these values represent upper-bound estimates under the two-gate case-control design with symptomatic controls, affected by spectrum bias, and additionally constrained by the study sample size. Bootstrap-optimism-adjusted C-statistic: 0.998 (500 resamples). Incorporation bias was specifically mitigated by excluding stool antigen from the case-definition reference standard.

Table 2. Within-sample diagnostic accuracy metrics (upper-bound case-control estimates — NOT expected real-world performance).

Biomarker	AUC (95% CI)	Sensitivity	Specificity	PPV*	NPV*	Youden Threshold
TNF-α	1.000 (1.000–1.000)	1.000	1.000	0.976	1.000	22.4 pg/mL
IL-8	1.000 (1.000–1.000)	1.000	1.000	0.976	1.000	84.6 pg/mL
Total IgA	1.000 (1.000–1.000)	1.000	1.000	0.976	1.000	2.14 mg/dL
Stool Antigen	1.000 (1.000–1.000)	1.000	1.000	0.976	1.000	48.3 S/CO

Note. *PPV/NPV at study-sample prevalence 66.7%. At clinical prevalence 10–30%, PPV falls to approximately 0.70–0.88 while NPV remains >0.99. These values are within-sample upper-bound estimates and should not be extrapolated to clinical practice without prospective validation in a consecutive patient series.

3.4 Disease Burden Stratification and TNF-α/IL-8 Ratio

Stool antigen (S/CO) was the only biomarker to demonstrate a statistically significant gradient across disease burden strata defined by the updated Sydney System histological grade (mild/moderate/severe; Kruskal-Wallis $p<0.001$; all Bonferroni-corrected pairwise comparisons significant). Serum TNF-α, IL-8, and total IgA showed no statistically significant variation across histological strata (all $p>0.05$), consistent with plateau-level cytokine activation once active mucosal inflammation is established. The TNF-α/IL-8 ratio was stable across all severity strata (mild: 1.14±0.09; moderate: 1.16±0.11; severe: 1.18±0.10; Kruskal-Wallis $p=0.612$), indicating proportional NF-κB-mediated co-activation of these mediators independent of bacterial colonisation density (Table 3).

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able 3. Biomarker concentrations and TNF-α/IL-8 ratio across disease burden strata (Updated Sydney System histological grade; n=60 cases).

Biomarker	Mild — Grade I (n=20)	Moderate — Grade II (n=20)	Severe — Grade III (n=20)	KW p
Stool Antigen (S/CO)	70.5 ± 12.2	102.5 ± 8.6	137.2 ± 9.0	<0.001
TNF-α (pg/mL)	46.3 ± 9.8	48.9 ± 10.4	50.7 ± 11.2	0.418
IL-8 (pg/mL)	181.2 ± 38.4	187.6 ± 41.3	190.3 ± 44.7	0.524
Total IgA (mg/dL)	3.76 ± 0.68	3.86 ± 0.71	3.91 ± 0.74	0.487
TNF-α/IL-8 Ratio	1.14 ± 0.09	1.16 ± 0.11	1.18 ± 0.10	0.612

Note. KW = Kruskal-Wallis + Dunn-Bonferroni post-hoc pairwise comparisons. Stool antigen: all pairwise comparisons significant ($p<0.05$ after Bonferroni correction). Severity defined by updated Sydney System histological grade, independent of any biomarker measurement.

3.5 Inter-Biomarker Spearman Correlations

All inter-biomarker Spearman correlations within the infected group were statistically significant after Benjamini-Hochberg false discovery rate correction (all FDR-corrected $q<0.001$; Table 4). The strongest association was observed between IgA and IL-8 ($\rho=0.704$), mechanistically coherent with IL-8-driven mucosal neutrophilic recruitment promoting B-cell IgA class switching.[9] Stool antigen demonstrated the weakest correlations with serum markers ($\rho=0.391$–0.438), consistent with its role as a bacterial burden proxy rather than a host immune activation marker. All correlations are descriptive; causality cannot be inferred from cross-sectional data.

Table 4. Spearman inter-biomarker correlations in the infected group (n=60).

TNF-α	IL-8	Total IgA	Stool Antigen (S/CO)
—	ρ=0.681*	ρ=0.623*	ρ=0.412*
	—	ρ=0.704*	ρ=0.438*
		—	ρ=0.391*
			—

Note. *FDR-corrected $q<0.001$ (Benjamini-Hochberg method, which controls the false discovery rate, not the familywise error rate). All associations are descriptive correlations; causal inference is not warranted from these cross-sectional data.

3.6 Firth Penalised Logistic Regression

Complete distributional separation between cases and controls necessitated Firth penalised logistic regression.[16] After covariate adjustment for age and sex (both $p>0.05$), TNF-α emerged as the strongest independent predictor of confirmed *H. pylori* infection status (adjusted OR=15.8; 95% CI: 2.4–104.3; Firth $p=0.004$). The wide CI reflects genuine statistical uncertainty inherent to Firth-penalised estimation under complete separation at this sample size and should not be interpreted as evidence against biomarker relevance. The Hosmer-Lemeshow test was non-significant ($p>0.05$) within this sample; however, this test has limited power under small samples and complete separation, and is reported as a supplementary indicator only. Bootstrap-optimism-adjusted C-statistic: 0.998.

Table 5. Baseline participant characteristics.

Characteristic	H. pylori Cases (n=60)	Controls (n=30)	p
Age, years — mean ± SD	38.5 ± 11.2	36.8 ± 10.7	0.472†
Sex — Male, n (%)	35 (58.3%)	18 (60.0%)	0.831‡
Sydney Grade I (Mild), n	20	—	—
Sydney Grade II (Moderate), n	20	—	—
Sydney Grade III (Severe), n	20	—	—
^{13}C-UBT positive, n (%)	60 (100%)	0 (0%)	<0.001§
PPI use within 2 weeks, n	0	0	—

Note. †Wilcoxon rank-sum test. ‡Pearson χ^2 test. §Fisher's exact test. Controls: symptomatic patients with upper GI complaints and negative ^{13}C-UBT and stool antigen ELISA. Sydney grade assigned by histopathologist blinded to biomarker data. PPI = proton pump inhibitor.

4. DISCUSSION

4.1 Interpretive Context: Design Limitations and Bias Mitigation

The central finding — complete within-sample distributional separation (AUC=1.000; r=1.00) — must be interpreted within the explicit limitations of the two-gate case-control design before any biological or clinical conclusions are drawn. Two specific design-inherent biases require direct acknowledgement.

First, spectrum bias: the two-gate design compares confirmed cases at peak immunological activation against screened controls, which systematically inflates apparent discriminatory performance relative to what would be observed in a consecutive series of symptomatic patients presenting for diagnostic evaluation.[17] Empirical data indicate AUC inflation of 0.05–0.15 units compared with prospective single-gate designs. AUC=1.000 should therefore be understood as an upper bound under maximally favourable design conditions, not as an expected real-world diagnostic accuracy figure. To partially mitigate spectrum bias, the present study recruited symptomatic controls (upper gastrointestinal complaints with negative reference tests) rather than purely asymptomatic volunteers.

Second, incorporation bias: this occurs when an index test (biomarker under evaluation) is included in the reference standard used to define case status, thereby artificially inflating sensitivity and specificity.[18,28] In the present study, stool antigen ELISA was specifically excluded from the case-definition reference standard (which comprised ^{13}C-UBT and endoscopic biopsy/histopathology/RUT only) to prevent this bias. Furthermore, disease burden severity was stratified using the updated Sydney System histological grade — an index entirely independent of the stool antigen measurement — preventing the circular inference that would have arisen had stool antigen tertiles been used as severity strata.

4.2 TNF-α as the Dominant Independent Predictor

TNF-α's dominance as the strongest Firth-corrected predictor (adjusted OR=15.8, p=0.004) is mechanistically consistent with the ubiquitous distribution of TNFR1, which produces broader systemic inflammatory signalling than the predominantly mucosal, CXCR1/CXCR2-dependent IL-8 response.[5,7] These findings align with prior reports of elevated serum TNF-α in *H. pylori*-positive patients correlating with histological inflammatory grade[19] and with multivariate cytokine analyses identifying TNF-α as a superior discriminator over IL-6 and IL-1β.[20] The wide Firth CI (2.4–104.3) reflects genuine statistical uncertainty under complete separation and reinforces the need for prospective validation with a larger sample.

4.3 IL-8–IgA Co-regulation and Regional Context

The strongest inter-biomarker correlation (IgA–IL-8, ρ=0.704) is mechanistically coherent: IL-8-driven mucosal neutrophilic recruitment promotes B-cell differentiation and local IgA class switching in the gastric lamina propria.[9] The median IL-8 in the present Iraqi cohort (186.4 pg/mL) is substantially higher than values reported in Thai (120–150 pg/mL) and Brazilian cohorts,[21,22] and closely aligned with Mohanad et al. (174.9±78.9 pg/mL; Al-Diwaniyah, Iraq).[23] Cross-study comparisons must be interpreted with caution given differences in ELISA kits, dilution factors, and patient populations. Virulence genotyping data are unavailable in the present study and represent a priority for future Iraqi cohort investigations.

4.4 Stool Antigen as a Severity-Responsive Biomarker

Stool antigen (S/CO) showed the only statistically significant gradient across Sydney-graded histological severity strata ($p<0.001$), whereas serum cytokines and IgA showed no significant variation across strata (all $p>0.05$). This pattern is mechanistically explicable: stool antigen scales with bacterial colonisation density as a direct antigen-shedding proxy, whereas cytokines likely plateau at near-maximal activation once mucosal inflammation is fully established.[4,9] Critically, because severity was defined by an independent histological index (Sydney System), this gradient represents a genuine association between stool antigen S/CO and gastric mucosal burden, not a circular artefact. If confirmed prospectively, serial stool antigen monitoring may provide real-time information on bacterial clearance during eradication therapy complementary to cytokine profiling.

4.5 Limitations

Beyond the design limitations discussed in Section 4.1, additional study-level limitations include: (1) single-centre recruitment from Samarra limits generalisability; (2) virulence genotyping (cagA, vacA) was not performed; (3) no incremental diagnostic value analysis (NRI/IDI, decision curve analysis) was conducted relative to standard-of-care tests; (4) the stool antigen ELISA used is a qualitative kit validated for binary positivity; semi-quantitative S/CO expression requires further validation against clinically validated quantitative assays; (5) the sample size (n=90), although meeting the a priori threshold, supports estimation of ORs with wide CIs under complete separation — larger prospective studies are required.

5. CONCLUSION

This case-control immunoprofiling study of 60 confirmed *H. pylori* cases and 30 symptomatic controls from Samarra, Iraq demonstrates that serum TNF-α, IL-8, total IgA, and stool antigen (S/CO) collectively characterise a distinct immunological signature of active infection, with complete within-sample distributional separation (AUC=1.000; optimism-adjusted C=0.998). These estimates represent upper-bound values under the two-gate design; spectrum bias and the limited sample size preclude direct generalisation. Incorporation bias was specifically mitigated by excluding stool antigen from the case-definition reference standard and by using an independent histological severity index. TNF-α is the strongest Firth-penalised independent predictor (OR=15.8; 95% CI 2.4–104.3; p=0.004). Stool antigen shows the only significant gradient across Sydney-graded severity strata, supporting its utility as a bacterial burden proxy. These findings constitute immunological Proof-of-Concept data. Multi-centre prospective validation in consecutive symptomatic patient series, incorporating virulence genotyping, validated clinical outcome endpoints, and formal incremental value analysis (NRI/IDI, DCA), is required before any clinical application of this biomarker panel.

AUTHOR CONTRIBUTIONS

H.H.I.: Conception, design, laboratory analysis, statistical analysis, manuscript writing, correspondence. S.N.H.: Patient recruitment, specimen collection, data entry. M.S.M.: Laboratory assays, data analysis, critical revision. All authors contributed to critical revision and approved the final version. All agree to be accountable for all aspects of the work (ICMJE 2024 criteria).

CONFLICTS OF INTEREST

None declared by any author.

FUNDING

No external funding. Conducted with departmental resources at Tikrit Teaching Hospital, Salah Al-Deen Directorate of Health, Ministry of Health, Iraq. No commercial reagent discounts or in-kind support were received.

DATA AVAILABILITY

Anonymised raw data (biomarker concentrations, histological grades, demographic variables) are available from the corresponding author upon reasonable request, subject to institutional ethical approval of the requesting organisation. Data will be shared in Microsoft Excel format within 30 days of a reasonable verified request.

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