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TNF-α-308 G/A POLYMORPHISM AS A GENETIC DETERMINANT OF SUSCEPTIBILITY TO *HELICOBACTER PYLORI* AND *CRYPTOSPORIDIUM* CO-INFECTION

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ABSTRACT

Tumour necrosis factor-alpha (TNF-α) is a key pro-inflammatory cytokine involved in host defence against gastrointestinal pathogens. This study investigated the association between the TNF-α −308 G>A (rs1800629) polymorphism and susceptibility to concurrent *Helicobacter pylori* and *Cryptosporidium* infection in an Iraqi population. A hospital-based case-control study included 100 unrelated Iraqi Arabs comprising 50 patients with confirmed co-infection and 50 healthy controls. Genotyping was performed using Tetra-Primer ARMS-PCR, and associations were analysed using logistic regression adjusted for age and sex. The control group conformed to Hardy-Weinberg equilibrium ($p = 0.716$). The AA genotype was significantly more frequent among co-infected patients than controls (28.0% vs. 4.0%; adjusted OR = 8.10, 95% CI: 2.95–22.24; $p < 0.001$), whereas the GA genotype showed no significant association. The A allele was associated with increased odds of co-infection (OR = 2.92, 95% CI: 1.52–5.60; $p = 0.0008$), and the recessive model demonstrated the strongest association (OR = 9.33; $p < 0.001$).
These findings provide novel evidence that the TNF-α −308 A allele is associated with increased odds of *H. pylori* and *Cryptosporidium* co-infection, highlighting the role of host genetic variation in polymicrobial gastrointestinal disease.

Keywords: TNF-α; *Helicobacter pylori*; *Cryptosporidium*; Genetic Susceptibility; Polymorphism; Co-Infection; rs1800629

INTRODUCTION

Tumour necrosis factor-alpha (TNF-α) is a pleiotropic pro-inflammatory cytokine that plays a central role in innate and adaptive immunity by regulating leukocyte recruitment, antimicrobial activity, and mucosal barrier integrity. Owing to its pivotal immunoregulatory functions, genetic variations within the TNF-α gene have been extensively investigated as determinants of susceptibility to infectious and inflammatory diseases **(Bradley, 2008; Qidwai & Khan, 2011)**.
The TNF-α gene is located on chromosome 6p21.3 within the major histocompatibility complex (MHC) class III region. Among the functional variants identified in its promoter region, the −308 G>A polymorphism (rs1800629) is the most extensively characterised. The A allele has been associated with enhanced promoter activity and approximately 6- to 7-fold higher TNF-α production than the G allele **(Kroeger *et al.*, 1997; Wilson *et al.*, 1997)**. Given the critical role of TNF-α in mucosal immunity and inflammatory regulation, this polymorphism may influence host susceptibility to gastrointestinal pathogens and polymicrobial infection.
Helicobacter pylori and *Cryptosporidium* spp. are among the most prevalent gastrointestinal pathogens worldwide and remain major causes of morbidity, particularly in low- and middle-income countries. *H. pylori* is primarily transmitted via person-to-person spread through oral–oral and faecal–oral routes, whereas *Cryptosporidium* infection is acquired mainly through ingestion of environmentally resistant oocysts in contaminated water or food and by faecal–oral transmission **(Checkley *et al.*, 2015; Hooi *et al.*, 2017)**. Because these pathogens share faecal–oral transmission pathways and common environmental and socioeconomic risk factors, concurrent infection may occur more frequently than previously recognised. Furthermore, altered mucosal immunity and impaired epithelial barrier function may facilitate pathogen coexistence and contribute to more severe gastrointestinal disease **(Hoarau *et al.*, 2020)**.
In Iraq, both pathogens represent important public health concerns. *Cryptosporidium* prevalence has been reported to range from 10.4% to 64.5% in different Iraqi populations, while *H. pylori* infection affects approximately 50–70% of individuals (**Salim & Al-Aboody, 2019**; **Alsafi & AL-Aboody, 2023; Marzoog & Salih, 2025; Ahmed & Muhaidi, 2026**). Despite this substantial epidemiological burden, most Iraqi studies have relied on conventional microscopy, serological assays, or rapid diagnostic tests. Although *H. pylori*–*Cryptosporidium* co-infection has previously been reported in Iraq, available investigations have been largely descriptive and have not incorporated molecular confirmation or host genetic analysis (**Rahi & Fadhil, 2021**). Furthermore, while the TNF-α rs1800629 polymorphism has been examined in Iraqi patients with non-infectious conditions such as prostate tumours and beta-thalassaemia major (**Ali *et al.*, 2019**; **Luaibi & Mohammed, 2023**), its role in gastrointestinal infections remains unexplored. Notably, no previous Iraqi study has simultaneously confirmed *H. pylori* and *Cryptosporidium* co-infection using molecular methods, genotyped the functional TNF-α −308 G>A (rs1800629) polymorphism, and evaluated its association with polymicrobial gastrointestinal infection. Therefore, the present study was designed to address this knowledge gap and represents the first Iraqi investigation integrating molecular diagnosis of *H. pylori*–*Cryptosporidium* co-infection with immunogenetic analysis of TNF-α rs1800629 susceptibility.

MATERIAL AND METHODS

Study Design and Participants

A hospital-based case-control study was conducted in Thi-Qar Province, southern Iraq, between January and April 2026. The study population comprised 100 participants, including 50 patients with confirmed concurrent *Helicobacter pylori* and *Cryptosporidium* infection and 50 apparently healthy controls. All participants were unrelated Iraqi Arabs residing in Thi-Qar Province.

Ethical Considerations

The study protocol was approved by the Research Ethics Committee of the Thi-Qar Health Directorate, Ministry of Health, Iraq (Approval No. 2026/27; January 27, 2026), and by the Ethical Committee of the College of Science, University of Thi-Qar (Reference No. 97; January 20, 2026). Written informed consent was obtained from all participants prior to enrolment. All procedures were conducted in accordance with the ethical principles of the Declaration of Helsinki.

Determination of Infection Status

Co-infection was defined as the concurrent presence of both *H. pylori* and *Cryptosporidium* infection in the same individual.
For the detection of *Cryptosporidium*, stool specimens were initially examined microscopically using the modified Ziehl–Neelsen acid-fast staining technique according to **Garcia (2007)**. Subsequently, nested polymerase chain reaction (PCR) targeting the small-subunit ribosomal RNA (SSU rRNA) gene was performed as previously described by **Xiao *et al.* (1999)**. Individuals were

considered positive for *Cryptosporidium* infection if either diagnostic method yielded a positive result.

For the detection of *H. pylori*, stool samples were initially screened using a stool antigen assay (Premier Platinum HpSA; Meridian Bioscience, USA). Subsequently, conventional PCR targeting the 16S rRNA gene was performed. Individuals were considered positive for *H. pylori* infection if either diagnostic method yielded a positive result.

Cases were defined as individuals who tested positive for both *Cryptosporidium* and *H. pylori* according to the above diagnostic criteria and were therefore classified as having *H. pylori–Cryptosporidium* co-infection. The control group comprised apparently healthy individuals who tested negative for both pathogens using the same diagnostic procedures.

Blood Sample Collection and DNA Extraction

Approximately 3 mL of peripheral venous blood was collected from each participant into EDTA-containing tubes. Genomic DNA was extracted from 200 µL of whole blood using the gSYAN™ DNA Blood Extraction Kit (Geneaid, Taiwan) according to the manufacturer's instructions. DNA concentration and purity were evaluated using a NanoDrop™ 2000c spectrophotometer (Thermo Scientific, USA). Samples with A260/A280 ratios ranging from 1.8 to 2.0 and DNA concentrations exceeding 20 ng/µL were considered suitable for subsequent analyses. Extracted DNA samples were stored at −20 °C until further use.

Genotyping of TNF-α −308 G>A (rs1800629) by Tetra-Primer ARMS-PCR

Primer Design and PCR Amplification

Tetra-Primer Amplification Refractory Mutation System Polymerase Chain Reaction (Tetra-Primer ARMS-PCR) primers for the TNF-α rs1800629 polymorphism were designed using an online primer design platform according to the method of **Collins and Ke (2012)**, based on reference sequences retrieved from the NCBI dbSNP database. The primer sequences and expected amplicon sizes are presented in Table 1.

Table 1 Tetra-Primer ARMS-PCR primers used for genotyping the TNF-α −308 G>A (rs1800629) polymorphism

Primer Type	Primer Name	Sequence (5′–3′)	Product Size
Inner Forward (G allele)	TNF-α FI	GGAGGCAATAGGTTTTGAGGGGCAGGG	198 bp
Inner Reverse (A allele)	TNF-α RI	GTAGGACCCTGGAGGCTGAACCCCGTACT	162 bp
Outer Forward	TNF-α FO	AGGACTCAGCTTTCTGAAGCCCCTCCCA	304 bp
Outer Reverse	TNF-α RO	TTCTGTCTCGGTTTCTTCTCCATCGCGG	304 bp

PCR amplification was carried out in a final reaction volume of 25 µL containing 5 µL of genomic DNA template, 1 µL of each primer (10 pmol/µL), 12.5 µL of GoTaq® G2 Green Master Mix (2×) (Promega, Korea), and 3.5 µL of nuclease-free water. The thermal cycling protocol consisted of an initial denaturation step at 95 °C for 5 min, followed by 35 amplification cycles of denaturation at 95 °C for 30 s, annealing at 62 °C for 30 s, and extension at 72 °C for 30 s, with a final extension step at 72 °C for 5 min. Positive and negative controls were included in each PCR run.

Genotype Determination and Quality Control

PCR products were separated by electrophoresis on 2.5% agarose gels stained with ethidium bromide and run at 100 V for 45 min. Amplified fragments were visualised under ultraviolet illumination. Genotypes were assigned according to the observed banding patterns as follows: GG genotype (304 bp and 198 bp), AA genotype (304 bp and 162 bp), and GA genotype (304 bp, 198 bp, and 162 bp). To assess genotyping reproducibility, 20% of the samples were randomly selected and re-analysed in a blinded manner, yielding 100% concordance.

Statistical Analysis

Statistical analyses were performed using IBM SPSS Statistics version 26.0 (IBM Corp., Armonk, NY, USA). Continuous variables were expressed as mean ± standard deviation (SD) and compared using the Mann–Whitney U test due to violation of normality assumptions, as assessed by the Shapiro–Wilk test ($p < 0.05$). Categorical variables were analysed using the chi-square test or Fisher's exact test, as appropriate. Hardy–Weinberg equilibrium (HWE) in the control group was evaluated using the chi-square goodness-of-fit test. Associations between the TNF-α rs1800629 polymorphism and susceptibility to co-infection were examined under genotypic, dominant (GA + AA vs. GG), recessive (AA vs. GG + GA), and allelic (A vs. G) inheritance models using logistic regression analyses. Odds ratios (ORs) and corresponding 95% confidence intervals (CIs) were calculated. To account for multiple comparisons among the four genetic models, Bonferroni correction was applied, and statistical significance for these genetic association analyses was set at $\alpha = 0.0125$. For the multivariable logistic regression model, which included covariates (age and sex), the conventional significance level of $\alpha = 0.05$ was retained. Multivariable logistic regression analyses were subsequently performed with adjustment for age and sex. The linearity assumption for age was evaluated using the Box-Tidwell test, which confirmed a linear relationship between age and the logit of the outcome ($p > 0.05$). Model calibration was assessed using the Hosmer–Lemeshow goodness-of-fit test, and the proportion of explained variance was estimated using Nagelkerke's R^2 statistic. All statistical tests were two-tailed, and $p < 0.05$ was considered statistically significant unless otherwise specified.

RESULTS AND DISCUSSION

Demographic Characteristics of the Study Population

A total of 100 unrelated Iraqi Arab individuals were enrolled in the present hospital-based case-control study, comprising 50 patients with confirmed concurrent *Helicobacter pylori* and *Cryptosporidium* infection and 50 apparently healthy controls. The demographic characteristics of the study population are summarized in Table 2.

Co-infected patients were significantly older than healthy controls (median age 35 years vs. 19 years, respectively; $p = 0.024$). This finding is biologically plausible because increasing age is associated with cumulative exposure to environmental pathogens and prolonged opportunities for acquiring gastrointestinal infections. Moreover, age-related alterations in mucosal immunity may contribute to the persistence of multiple pathogens and facilitate polymicrobial interactions **(Hoarau *et al.*, 2020)**.

No significant difference in sex distribution was observed between cases and controls ($p = 1.00$), indicating that the study groups were comparable with respect to sex and reducing the likelihood of sex-related confounding in the subsequent genetic analyses.

Table 2 Demographic characteristics of the study population

Characteristic	Controls (n = 50)	Co-infected patients (n = 50)	Total (n = 100)	*p*-value
Age (years), mean ± SD	22.4 ± 21.3	34.6 ± 13.2	27.8 ± 16.2	0.024*
Median (IQR)	19 (13–28)	35 (25–44)	26 (18–37)	
Male, n (%)	21 (42.0)	21 (42.0)	42 (42.0)	1.00†
Female, n (%)	29 (58.0)	29 (58.0)	58 (58.0)	

*Mann-Whitney U test. †Chi-square test.

Distribution of TNF-α −308 G>A Genotypes and Alleles

To assess the quality of genotyping and the absence of population stratification, Hardy–Weinberg equilibrium (HWE) was tested in the control group. The observed genotype frequencies (GG = 34, GA = 14, AA = 2) did not deviate

significantly from those expected under HWE (GG = 33.62, GA = 14.76, AA = 1.62; $\chi^2 = 0.132$, df = 1, p = 0.716; exact p = 0.722 due to low expected count for the AA genotype), as shown in Table 3. This finding supports the reliability of the genotyping procedure and indicates the absence of substantial population stratification or major genotyping errors (**Namipashaki *et al.*, 2015**).

Table 3 Hardy–Weinberg equilibrium in the final control cohort (n = 50)

Genotypes	Observed (n)	Expected (n)	χ^2 contribution	df	p-value
GG (homozygote reference)	34	33.62	0.004	1	0.716
GA (heterozygote)	14	14.76	0.039		
AA (homozygote variant)	2	1.62	0.089		
Total	**50**	**50.00**	**0.132**		

Chi-square test: $\chi^2 = 0.132$, df = 1, p = 0.716. Exact p = 0.722. Non-significant (p > 0.05).

Following the confirmation of HWE, the association between TNF-α rs1800629 genotypes and susceptibility to *H. pylori–Cryptosporidium* co-infection was evaluated. A significant difference in genotype distribution was observed between cases and controls (Table 4). The AA genotype was markedly overrepresented among co-infected patients compared with controls (28.0% vs. 4.0%), corresponding to an odds ratio of 9.52 (95% CI: 2.83–32.04; p < 0.001). In contrast, the heterozygous GA genotype showed no significant association with susceptibility (p = 0.99). The A allele was also significantly more frequent among co-infected patients than controls (39.0% vs. 18.0%; OR = 2.92, 95% CI: 1.52–5.60; p = 0.0008).

Table 4 Distribution of TNF-α −308 G>A (rs1800629) genotypes and alleles according to infection status

Group	GG, n (%)	GA, n (%)	AA, n (%)	Total	A allele (%)
Controls	34 (68.0)	14 (28.0)	2 (4.0)	50	18.0
Co-infected patients	25 (50.0)	11 (22.0)	14 (28.0)	50	39.0
OR (95% CI)	Reference	1.07 (0.42–2.72)	9.52 (2.83–32.04)		2.92 (1.52–5.60)
p-value		0.99	<0.001*		0.0008*

*Significant after Bonferroni correction (α = 0.0125).

Genetic Models and Biological Interpretation

Analysis under different inheritance models demonstrated that the dominant model (GA + AA vs. GG) was not statistically significant (OR = 2.13; p = 0.069). In contrast, the recessive model (AA vs. GG + GA) showed a strong association with susceptibility to co-infection (OR = 9.33, 95% CI: 2.72–32.01; p < 0.001).

These findings may suggest that the biological effect of the −308A allele becomes evident primarily in homozygous carriers, indicating that homozygosity for the A allele may be required for the effect to manifest.

This observation is biologically plausible because the −308A allele has been shown to increase TNF-α promoter activity and results in approximately 6- to 7-fold higher constitutive and inducible TNF-α expression than the common G allele **(Wilson *et al.*, 1997)**. Excessive TNF-α production may disrupt mucosal homeostasis, amplify inflammatory tissue injury, and impair the coordinated immune responses required to control both *H. pylori* and *Cryptosporidium* infection **(Sharifi *et al.*, 2022)**.

These findings agree with previous studies reporting associations between TNF-α genetic variation and susceptibility to *H. pylori* infection and related gastrointestinal disorders **(Zhao *et al.*, 2013)**. Likewise, elevated TNF-α expression has been documented during cryptosporidiosis and may contribute to intestinal epithelial dysfunction and increased inflammatory damage **(Lean *et al.*, 2006; de Sablet *et al.*, 2016; Amin *et al.*, 2021)**. Collectively, these observations suggest that genetically determined dysregulation of TNF-α production may be associated with persistent or simultaneous gastrointestinal infections.

ARMS-PCR Genotyping

Representative Tetra-Primer ARMS-PCR products are presented in Figure 1. Successful amplification was confirmed by the presence of the 304-bp control fragment in all positive samples. Genotypes were clearly distinguished by allele-specific amplicons, demonstrating the reliability and suitability of the Tetra-Primer ARMS-PCR assay for genotyping the TNF-α −308 G>A polymorphism.

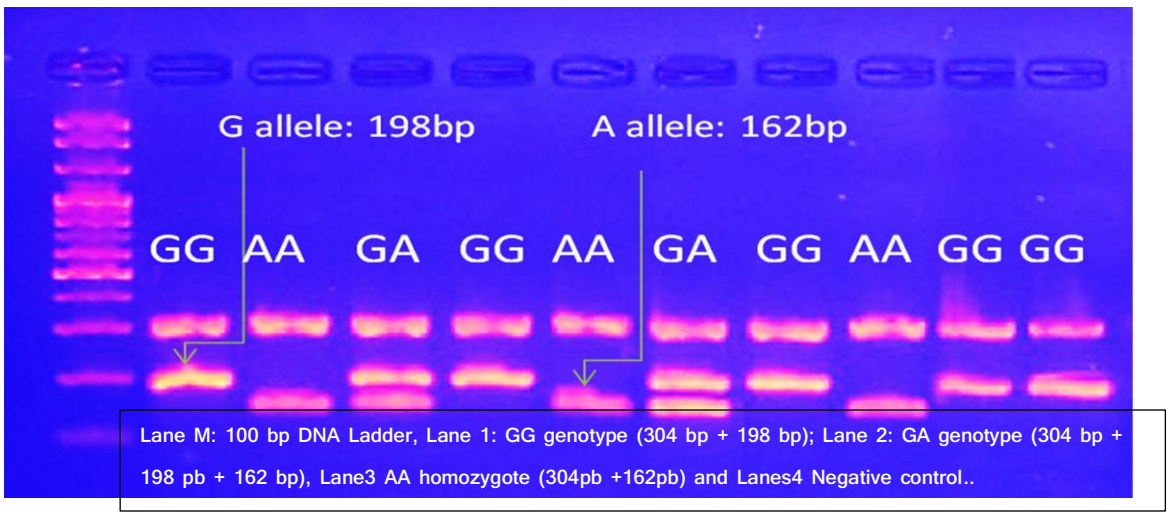

Figure 1 Representative agarose gel electrophoresis of Tetra-Primer ARMS-PCR products used for genotyping the TNF-α −308 G>A (rs1800629) polymorphism. Lane M: 100-bp DNA ladder; Lane 1: GG genotype (304 and 198 bp); Lane 2: GA genotype (304, 198, and 162 bp); Lane 3: AA genotype (304 and 162 bp); Lane 4: negative control.

Multivariable Logistic Regression Analysis

To determine whether the observed genetic association was independent of demographic characteristics, multivariable logistic regression analysis was performed (Table 5).

After adjustment for age and sex, the AA genotype remained the strongest independent predictor of co-infection susceptibility (adjusted OR = 8.10, 95% CI: 2.95–22.24; $p < 0.001$). Age showed a modest but statistically significant effect ($p = 0.048$), whereas sex was not associated with disease status.

The regression model demonstrated acceptable calibration (Hosmer–Lemeshow $p = 0.58$) and explained approximately 38% of the variability in co-infection status (Nagelkerke $R^2 = 0.38$). The persistence of the genetic association after adjustment for potential confounders strengthens the evidence that the TNF-α −308 AA genotype may represent an independent genetic determinant of susceptibility to concurrent *H. pylori* and *Cryptosporidium* infection.

Table 5 Multivariable logistic regression analysis of factors associated with co-infection

Variable	Adjusted OR	95% CI	*p*-value
TNF-α AA genotype vs. GG + GA	8.10	2.95–22.24	<0.001
Age (per year)	1.03	1.00–1.06	0.048
Male sex	0.89	0.41–1.93	0.762

Model statistics: Hosmer–Lemeshow *p* = 0.58; Nagelkerke $R^2 = 0.38$.

Clinical and Epidemiological Implications

The present study addresses an important gap in the literature by examining susceptibility to polymicrobial gastrointestinal infection rather than single-pathogen infection. Because *H. pylori* and *Cryptosporidium* share common environmental exposures and faecal–oral transmission pathways, host genetic factors regulating mucosal immunity may influence susceptibility to concurrent infection **(Hoarau *et al.*, 2020)**.

The identification of the TNF-α −308 AA genotype as an independent risk factor suggests that genetically determined high TNF-α production may contribute to susceptibility to co-infection and may eventually facilitate risk stratification and targeted surveillance in high-risk populations if validated in larger studies.

Strengths and Limitations

Strengths

This study has several strengths. First, it is the first investigation in Iraq to integrate molecular confirmation of *H. pylori–Cryptosporidium* co-infection with genotyping of the functional TNF-α rs1800629 polymorphism. Second, infection status was established using complementary diagnostic approaches, including microscopy, stool antigen testing, and PCR-based methods, thereby reducing the likelihood of disease misclassification. Third, genotyping quality was supported by blinded repeat analyses with complete concordance and by conformity with Hardy–Weinberg equilibrium. Finally, the application of Bonferroni correction and multivariable logistic regression strengthened the robustness and validity of the statistical inferences.

Limitations

Several limitations should be acknowledged. The relatively modest sample size limited statistical power for detecting weaker genetic effects and resulted in relatively wide confidence intervals. A post hoc power calculation for the observed AA genotype effect (OR = 9.52) indicated power > 80% at $\alpha = 0.05$; however, this estimate should be interpreted cautiously given the wide confidence intervals. The hospital-based, single-centre design may restrict the generalisability of the findings to other Iraqi populations. In addition, data regarding socioeconomic status, sanitation practices, drinking water sources, household crowding, educational level, and previous antimicrobial treatment were not collected. These factors are known to influence susceptibility to both *H. pylori* and *Cryptosporidium* infection, and their absence may result in residual confounding. Circulating TNF-α concentrations and functional assays were not performed; therefore, the mechanistic consequences of the observed genetic associations could not be directly evaluated. Furthermore, only a single TNF-α polymorphism was investigated, and potential interactions with other host genetic variants and environmental factors warrant further investigation in larger multicentre studies.

CONCLUSION

In conclusion, this study provides the first molecular and immunogenetic evidence from Iraq demonstrating a significant association between the functional TNF-α −308 G>A (rs1800629) polymorphism and *H. pylori–Cryptosporidium* co-infection status. Specifically, the AA homozygous genotype and the A allele were significantly associated with increased odds of co-infection, with the strongest association observed under a recessive genetic model.

By integrating molecular confirmation of polymicrobial gastrointestinal infection with host genetic analysis, this study extends current understanding of the role of TNF-α-mediated immune responses in determining susceptibility to concurrent enteric infections. Given that both pathogens share common environmental exposures and faecal–oral transmission pathways, the findings suggest that genetically determined variation in inflammatory responses may influence the establishment and persistence of polymicrobial gastrointestinal infections.

These findings contribute novel baseline data for the Iraqi and Middle Eastern populations and provide a foundation for future multicenter and functional studies investigating cytokine-mediated mechanisms of susceptibility, risk stratification, and the potential incorporation of host genetic markers into precision approaches for the prevention and management of gastrointestinal co-infections.

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