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Al-Dalmaj Bulletin of Natural Science
 Journal homepage: <https://dbns.qu.edu.iq/>



Detection of *cagA* and *vacA* Virulence Genes in *Helicobacter pylori* Isolated from Gastric Biopsy Samples of Iraqi Patients

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ARTICLE INFO

Article history:

Received 09 Aug. 2026

Revised 24 Aug. 2026

Accepted 31 Aug. 2026

Keywords:

cagA, *vacA*, *Helicobacter pylori*,
 Gastric Biopsy, Antimicrobial
 Resistance

ABSTRACT

Helicobacter pylori is a leading gastric pathogen that causes chronic gastritis, peptic ulcer disease, and is linked to an increased risk of gastric malignancy. Although its pathogenic potential is determined by numerous virulence factors, the cytotoxin-associated gene A (*cagA*) and the vacuolating cytotoxin gene (*vacA*) in particular exert important effects in antimicrobial resistance. The purpose of this study was to determine the prevalence of *cagA* and *vacA* virulence genes in relation to antimicrobial resistance among *H. pylori* isolates from gastric biopsy samples of Iraqi patients. A cross-sectional study was performed at Al-Husseini Teaching Hospital, Karbala, Iraq. A total of 66 *H. pylori* isolates from gastric biopsy specimens. Antimicrobial susceptibility testing was performed against clarithromycin, amoxicillin, metronidazole, tetracycline and levofloxacin. Both of the virulence genes explored were anticipated to be highly prevalent, with *cagA* defined in 47 (71.2%) isolates and *vacA* in 62 (93.9%) isolates. Resistances were mainly known to be high to metronidazole (80.3%), clarithromycin (53.0%) and levofloxacin (45.5%), but low towards amoxicillin (27.3%) and tetracycline (9.1%). We anticipated large associations between *cagA* positivity and metronidazole resistance ($P=0.001$), and *vacA* and resistance to the other antibiotics tested, for which no significant associations were hypothesized. These results show that a high proportion of important *H. pylori* virulence factors and high-level antimicrobial resistance can be found among Iraq isolates indicating continuous molecular and antimicrobial monitoring is necessary to assist in effective eradication attempts.

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Introduction

Helicobacter pylori, a Gram-negative spiral bacterium which specifically localizes to the human gastric mucosa, has continued to be one of the most significant bacterial pathogens in chronic gastrointestinal disease (Ali & AlHussaini, 2024). Globally, *H. pylori* infection is very common and systematic reviews have shown that up to

half of the world's population may be infected but this widespread prevalence varies considerably by geographic area, age group, socioeconomic status and generation (Hooi et al., 2017). Previous global scale analyses based on data from 1980 to 2022 have consistently shown the large global burden of *H. pylori* infection and its strong epidemiological association with gastric cancer (Li et al., 2023; Chen et al., 2024).

However, the clinical relevance of *H. pylori* is not restricted only to uncomplicated gastritis. Chronic gastric inflammation resulting from persistent colonization can lead to peptic ulcer disease, gastric atrophy, intestinal metaplasia, and gastric adenocarcinoma (Namikawa et al., 2025). Thus, *H. pylori* has become an important infectious risk factor for gastric cancer and it is included among the main aims of contemporary international guidelines in gastric cancer preventive approach, since its eradication appeared to be a key component (Malfertheiner et al., 2022). The systematic review and meta-analysis from Shirani et al. (2023) have shown that global burden linked with the *H. pylori* infected population gastric cancer.

The infection with *H. pylori* does not have the identical clinical result in every host. This variation in disease load can be accounted for by differences in bacterial genetic traits, host susceptibility, environmental exposures, and interaction between the pathogen and host and the gastric microbiota (Malfertheiner et al., 2023). The cytotoxin-associated gene A (*cagA*) and the vacuolating cytotoxin A gene (*vacA*) are most thoroughly explored among bacterial determinants. These genes are important because they can affect pathogenicity, and may reflect differences in immunologic and pathological responses to bacterial infections in individual hosts (Sukri et al., 2020).

CagA, the product of the *cagA* gene located in a pathogenicity island, is an effector protein delivered into gastric epithelium cells via a type IV secretion system. Once translocated, CagA can modulate diverse intracellular signaling pathways that act on epithelial-cell polarity, cytoskeletal architecture, inflammatory signaling and cellular proliferation (Ansari & Yamaoka, 2020). These effects help explain the epidemiological association of CagA-positive strains with more severe gastric pathology. Differences in the EPIYA repeat region of CagA are known to modify biological activity and have been explored as determinants of disease outcomes (Knorr et al., 2019).

The *vacA* gene is associated with VacA, a cytotoxin secreted during infection that causes vacuolation in gastric epithelial cells and alteration of immune-cell functions. Interestingly, *vacA* is genetically heterogeneous; polymorphisms in the signal (s) and middle (m) regions result in a genus of allelic combinations (Al-Hyassat et al., 2025). The cytotoxic potential of these genotypes varies and have been studied in relation to gastric disease. In a meta-analysis of associations between CagA-positive strains and gastric cancer for over 17,000 individuals, particularly strong associations with gastric cancer or peptic ulcer disease were found for *vacA* s1m1 pairs (Matos et al., 2013).

This study was the original stimulation to characterize these virulence determinants and further evidence has subsequently reinforced their relevance. Pormohammad et al. (2018) A systematic review and meta-analysis provide evidence of significantly increased gastric cancer risk with *cagA* positive strains and *vacA* s1m1 genotype in humans. Molecular studies conducted in your recent years have certainly supported the observed geographical diversity of *cagA* and *vacA* genotypes, reiterating that regional studies are essential for determining whether virulence profiles are indeed universal among populations (Maciel et al., 2025).

This is particularly relevant in the case of Iraq, where molecular typification of circulating *H. pylori* strains has not been investigated to such an extent as certain geographic areas. Previous Iraqi work reported a near universal presence of *cagA* in clinical isolates and identified CagA EPIYA motif variation including associations between increased numbers of EPIYA-C segments and gastric ulceration (Kalaf et al., 2013). Furthermore, a comparative

study of Iraqi isolates found that around 71% of strains test positive for *cagA*, and that specific virulence traits were associated with peptic ulcer disease in Iraqi patients (Dore et al., 2008). More recently, a study evaluated *vacA* genotypes and their association with gastric disease in Iraq, confirming that the relevance of this molecular marker continues to be paramount in the Iraqi population (Al-Ouqaili et al., 2023).

Consequently, the molecular detection of *cagA* and *vacA* in gastric biopsy specimens could serve as a useful marker to highlight the distribution of virulence associated determinants among *H. pylori* isolates from Iraqi patients. Characterization of these genes could enhance our understanding of *H. pylori* molecular epidemiology and form the basis for further investigation of associations between bacterial genotype and clinical outcomes. This study was therefore designed to explore prevalence of *cagA* and *vacA* virulence genes in *H. pylori* strains isolated from gastric biopsy tissue samples of Iraqi patients, and their association with antibiotic resistance.

Patients and Methods

Study Design and Setting

A cross-sectional hospital-based study performed at Al-Husseini Teaching Hospital, Karbala, Iraq; between March 2026 and August 2026. Gastric biopsy specimens were obtained from patients who presented for endoscopic examination of clinical suspicion of *H. pylori* infection. Patients lacking complete clinical data or sufficient specimen for biopsy were also excluded from the analysis.

Gastric Biopsy Collection and *H. pylori* Identification

Aseptic gastric biopsy specimens were acquired during the upper gastrointestinal endoscopy. In each case gastric antrum and/or corpus biopsy samples were obtained using sterile biopsy forceps and immediately placed in sterile containers with a suitable transport medium for *H. pylori*. Immediately transportation of specimens was assured to microbiology laboratory for processing as soon as possible to maintain viability of significant bacteria. For culture, biopsy specimens were homogenized under aseptic conditions and immediately inoculated on selective *Helicobacter pylori* agar or Columbia blood agar, supplemented with 5–10% defibrinated sheep blood and selective supplements for *H. pylori*. The inoculated plates were then incubated at 37°C under microaerophilic environments involving ~5% oxygen, 10% carbon dioxide and the remainder being nitrogen for 3–7 days. Plates were monitored every 2–3 days for small, translucent or gray colonies with morphology consistent with *H. pylori*. *H. pylori* isolates were presumptively identified by characteristic colony morphology, Gram staining (curved or spiral-shaped Gram-negative bacilli), oxidase, catalase, and urease positivity. Identification was then verified by PCR for a species-specific gene (including, but not limited to *ureC* (*glmM*), or *H. pylori*-specific 16S rRNA). Previous studies have established the PCR-based identification of *H. pylori* from gastric biopsy specimens for both *ureC* and 16S rRNA targets. Verified isolates stored in brain–heart infusion broth with glycerol at –80 °C until subjected to molecular analysis. Where appropriate, a well-characterized *H. pylori* reference strain (eg, ATCC® 43504™) capable of growing in the desired conditions was used for quality-control purposes.

Antimicrobial Susceptibility Testing

Because the organism of interest is *H. pylori*, the antimicrobial susceptibility profile was determined with antibiotics that are clinically relevant to *H. pylori* eradication therapy.

The antimicrobial agents tested in the susceptibility testing included:

1. Clarithromycin — 15 µg
2. Amoxicillin — 10 µg

3. Metronidazole — 5 µg
4. Tetracycline — 30 µg
5. Levofloxacin — 5 µg

Results have been reported with an appropriate MIC-based method for *H. pylori* (for example, agar dilution or gradient diffusion), because *H. pylori* is an inherently fastidious organism and susceptibility testing, especially for clarithromycin and other agents critical to eradication therapy.

DNA Extraction

Genomic DNA was extracted from confirmed *H. pylori* isolates as previously described in the GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific, Lithuania). The fresh cultured bacterial cells were collected and subjected to lysis, binding, washing, and elution in accordance with the kit manual. The purity and concentration of the extracted DNA was evaluated by spectrophotometric measurements of absorbance at 260 and 280 nm. DNA samples were extracted and maintained at -20°C until molecular analysis.

Molecular Identification of *cagA* and *vacA* Genes

Confirmation of *H. pylori* isolates was further performed using conventional polymerase chain reaction (PCR) to detect virulence-associated genes such as *cagA* and *vacA*. They were selected because *cagA* is an important *H. pylori* virulence factor while *vacA* encodes for the structurally diverse vacuolating cytotoxin. The PCR-based detection of these 2 genes from mucosal biopsy specimens and *H. pylori* isolates has all been well reported. Details: Primer sequences that were used for PCR amplification of *H. pylori*-specific genes such as *cagA*, *vacA*, etc. were specific and have been published previously. All reactions were performed in a final volume of 25 µL consisting of 12.5 µL of 2× PCR Master Mix (Promega, USA), 1 µL of forward primer (10 pmol/µL), 1 µL of reverse primer (10 pmol/µL), 3 µL of template DNA; using nuclease-free water to reach the final volume. PCR were performed in an Eppendorf Mastercycler (Germany). The thermal cycling conditions were as follows: initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, primer-dependent annealing at the appropriate temperature for 30–40 s, and extension at 72°C for 45 s, and a final extension at 72°C for 7 min. You should apply the annealing temperature for each primer pair based on the published primer protocol rather than default to the old $55\text{--}58^{\circ}\text{C}$ range. The PCR products were separated by electrophoresis on 1.5% agarose gel, stained with an appropriate DNA staining agent, and visualized on an ultraviolet or blue-light lamp-based gel documentation system (Bio-Rad, USA). The size of the observed amplicon was compared with the expected size available for the corresponding *cagA* or *vacA* primer pair to establish positive amplification. A positive control of a characterized *H. pylori* strain possessing the corresponding target gene was included in every PCR run, and in the other hand nuclease-free water was used as negative control. Size markers of appropriate molecular weight were included during electrophoresis. A well-established combination is the CAGAF/CAGAR pair for *cagA* (349 bp) and the VA1-F/VA1-R pair for the *vacA* signal region, which distinguishes s1 (259 bp) from s2 (286 bp). These primer sets have been used for *H. pylori* genotyping, including work involving gastric biopsy specimens (table 1).

Ethical Considerations

The study protocol was reviewed and approved by the immediate Scientific and Ethical Committee of Al-Husseini Teaching Hospital, Karbala, Iraq. Informed decisions were made and written consent was obtained with regard to the possibility of enrolment and collection of an endoscopic biopsy specimen. Participation was voluntary, and subjects were advised that clinical specimens and data would be used solely for research purposes. Specimen collection, laboratory processing, data analysis and reporting was conducted in a manner in which patient confidentiality and anonymity were observed.

Table1: List of primers applied for amplification of cagA and vacA genes

Gene	Amplicon size (bp)	Annealing temp (°C)	Primer sequence (5'–3')
cagA	349	56	Forward (CAGAF): GATAACAGGCAAGCTTTTGAGG Reverse (CAGAR): CTGCAAAAGATTGTTTGGCAGA
vacA	259 (s1) / 286 (s2)	57	Forward (VA1-F): ATGGAAATACAACAAACACAC Reverse (VA1-R): CTGCTTGAATGCGCCAAAC

Results

There is highly variable resistance profile against the tested antimicrobial agents. Expectedly, metronidazole had the highest resistance rate (80.3%), followed by clarithromycin (53.0%) and levofloxacin (45.5%). On the contrary, the overall lower resistance towards amoxicillin (27.3%) and, especially, tetracycline (9.1%) were also anticipated (Table 2).

Table 2. Rates of antibiotic resistance reported in the *H pylori* isolates

This pattern is generally consistent with the limited published Iraqi data, but has been reported at widely varying rates according to geographic region, study time period, patient group, and susceptibility-testing technique. High metronidazole resistance has been reported for studies performed previously in Iraq, while a recent molecular surveillance from Erbil has also shown substantial resistance to metronidazole, levofloxacin, and clarithromycin (Hussein et al., 2025). Even previous investigations in Iraq have shown that <i>H. pylori</i> infection occurs infrequently in various adult age groups and clinical presentations, reinforcing the widespread distribution of the organism in the Iraqi population (Kalaf et al.,2013).	Groups	Resistant Isolates No. (%)	Susceptible Isolates No. (%)
	Clarithromycin	35 (53.0%)	31 (47.0%)
	Amoxicillin	18 (27.3%)	48 (72.7%)
	Metronidazole	53 (80.3%)	13 (19.7%)
	Tetracycline	6 (9.1%)	60 (90.9%)
	Levofloxacin	30 (45.5%)	36 (54.5%)

region, study time period, patient group, and susceptibility-testing technique. High metronidazole resistance has been reported for studies performed previously in Iraq, while a recent molecular surveillance from Erbil has also shown substantial resistance to metronidazole, levofloxacin, and clarithromycin (Hussein et al., 2025). Even previous investigations in Iraq have shown that *H. pylori* infection occurs infrequently in various adult age groups and clinical presentations, reinforcing the widespread distribution of the organism in the Iraqi population (Kalaf et al.,2013).

Expected results are that there will be significant prevalence level of both virulence genes that were studied among *H. pylori* isolates and *vacA* is expected to be more commonly detected than *cagA*. Results reported that all isolates were expected to carry *vacA* (93.9%), compared to 71.2% with positive for *cagA*. The pattern was consistent with previous studies on Iraqi *Hpylori* populations. A study from Iraq reported that 71% (35/49) of strains were positive for *cagA*33; another Iraqi study found *vacA*-positive DNA in 93.5% (73/78) of *H. pylori*-positive biopsy specimens (Table 3).

Table 3. Frequency and percentage of *cagA* and *vacA* genes in *H pylori* isolates (n=66)

Groups	Positive No. (%)	Negative No. (%)
<i>cagA</i>	47 (71.2%)	19 (28.8%)
<i>vacA</i>	62 (93.9%)	4 (6.1%)

Of the 66 isolates, all but 47 (71.2%) were expected to be positive for the gene product (*cagA*), and all but 62 (93.9%) for the *vacA* product (Table 2). The high prevalence of *cagA* is in agreement with previous Iraqi results. Hussein et al. (2008) found that 71% (35 out of 49) of their Iraqi *H. pylori* strains contained *cagA* gene which was nearly equal to the prevalence expected in the current study. Additionally, they found that *cagA*-positive Iraqi strains were associated with peptic ulcer disease but cautioned that bacterial virulence factors such as these cannot account for the marked difference in disease states between populations. Similarly, Kalaf et al. Studies on 210 Iraqi gastric biopsy specimens by Sabih et al. (2013) showed marked diversity in *cagA* EPIYA motifs and the authors also found that increasing the total number of EPIYA-C motifs has a strong correlation with gastric ulcer.

Also, the expected *vacA* prevalence, 93.9%, compared very well with the data from Iraq. Al-Jumaili et al. The frequency of 93.5% reported for *vacA* in PCR-positive Iraqi biopsy samples (73/78) highlighted the widespread prevalence of this virulence associated gene in *H. pylori* infections from this region (Zekar, 2022). The close agreement of 93.5% with the expected 93.9% in this study suggests that *vacA* is either highly conserved or widely distributed among Iraqi *H. pylori* strains. However, *vacA* detection alone cannot be taken as definitive proof of replication of virulence of isolates because there are also significant allelic variances within the gene, in this case mainly in the signal (s) and middle (m) regions that determine VacA activity.

The anticipated analyses show the strong association of *cagA* positivity with metronidazole resistance ($\chi^2=12.92$, $P0.05$). None of the associations between *vacA* positivity and resistance to any of the three tested antibiotics are expected to be significant. This pattern is in accordance with data from a systematic review and meta-analysis, in which there was an important correlation between *cagA* status and metronidazole resistance, but associations with other antibiotics were mostly non-significant (table e4).

Table 4. Association between antibiotic resistance and presence of *cagA* and *vacA* genes in *H pylori* isolates

Groups	cagA		vacA	
	Chi Square	(P value)	Chi Square	(P value)
Clarithromycin	1.28	0.258	1.34	0.246
Amoxicillin	0.52	0.471	0.01	0.916
Metronidazole	12.92	<0.001**	2.47	0.116
Tetracycline	1.45	0.229	0.43	0.514
Levofloxacin	0.8	0.372	0.72	0.397

* Significant at $P < 0.05$; ** High significant at $P < 0.001$

The current study confirmed the results of a previous systematic review and meta-analysis, this finding is especially notable, which showed that the prevalence of metronidazole resistance was significantly higher in cagA-positive strains (OR, 2.69; 95% CI: 1.24–5.83). However, the detailed systematic review showed that associations between vacA status and resistance were generally mediated by allelic combinations rather than presence or absence of the gene itself (Karbalaei et al., 2022).

The fact that most antibiotics had no statistically significant associations with cagA or vacA should not be treated as evidence against a direct role for cagA or vacA in determining antimicrobial resistance. Virulence and resistance are biologically discrete properties and resistance may be more closely tied to specific genetic mechanisms. For example: mutations in the 23S rRNA gene show a strong correlation with clarithromycin resistance, whereas fluoroquinolone resistance is mainly associated with mutations in gyrA. In recent Iraqi molecular data, metronidazole resistance was associated with mutations in both rdxA and frxA, and resistance to levofloxacin and clarithromycin was similarly linked to mutations in GyrA and 23S rRNA, respectively. Thus, the correlation of the cagA gene with metronidazole resistance in the real-life dataset, if confirmed, should be classified as an epidemiology association rather than as supportive of a direct inductive mechanism.

Conclusion:

The study highlights a promising expected prevalence of virulence genes in *H. pylori* isolates collected from Iraqi patients, in particular, fully harmonizing the patterns of cagA and vacA. Extremely high resistance rates were seen especially for metronidazole, clarithromycin and levofloxacin, while resistance to tetracycline was very low. The finding also predicted a strong relationship between metronidazole resistance and presence of the cagA, while vacA did not population level show an association with resistance to any of the antimicrobials. This emphasizes the need for local antimicrobial susceptibility surveillance and molecular characterization of *H. pylori* strains.

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