ORIGINAL ARTICLE

Duodenal ulcer promoting gene 1 (*dupA1*) is associated with A2147G clarithromycin-resistance mutation but not interleukin-8 secretion from gastric mucosa in Iraqi patients

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Abstract

Helicobacter pylori causes peptic ulceration and gastric adenocarcinoma. The aims were to study the influence of *dupA1* positivity upon interleukin-8 (IL-8) secretion from gastric mucosa and determine the prevalence of mutations responsible for clarithromycin and fluoroquinolone resistance. DNA was extracted from 74 biopsies and the virulence factors were studied. Levels of IL-8 in gastric mucosa were measured using ELISA and the mutations responsible for clarithromycin and fluoroquinolone resistance were determined using a GenoType-HelicoDR assay. The prevalence of *cagA* in strains isolated from gastric ulcer (GU) and duodenal ulcer (DU) was significantly higher than those isolated from non-ulcer disease (NUD) (90% and 57.9% versus 33.3%; p 0.01). The *vacA* s1m1 genotype was more prevalent in patients with DU (73.7%) and GU (70%) than in those with NUD (13.3%) (p 0.01). The prevalence of *dupA1* was higher in DU patients (36.8%) than those with GU (10%) and NUD (8.9%) (p 0.01). Multivariate analysis showed that a *cagA*+/*vacA* s1i1m2 virulence gene combination was independently associated with the developing peptic ulcer disease (PUD) with increased odds of developing PUD (p 0.03; OR = 2.1). We found no significant difference in the levels of IL-8 secretion in gastric mucosa infected with *H. pylori dupA*-negative and *H. pylori dupA1*-positive strains (*dupA*-negative: mean ± median: 28 ± 26 versus 30 ± 27.1 for *dupA1*; p 0.6). While 12 strains were clarithromycin resistant, only three isolates were levofloxacin resistant. A significant association was found between *dupA1* genotype and A2147G clarithromycin resistance mutation (p <0.01). Further study is needed to explore the relationship between virulence factors and disease process and treatment failure.

Keywords: Antibiotic sensitivity, clarithromycin, *dupA*, fluoroquinolones, Iraq
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Introduction

Helicobacter pylori infection is associated with peptic ulcer disease (PUD) and gastric cancer. Virulence factors such as *cagA*, *vacA* and *dupA* play an important role in the pathogenesis of *H. pylori*-related diseases. Strains of *H. pylori* possessing *cagA* have been shown to associate with a significantly increased risk for the development of atrophic gastritis, peptic ulcer disease and gastric cancer [1]. The *vacA* and its polymorphism contribute significantly to the disease development process. According to the segment region (s) and middle region (m) the status of *vacA* can be classified into four subtypes: s1m1, s1m2, s2m1 and s2m2. *In vitro* experiments showed that s1m1 strains are the most cytotoxic, followed by s1m2 strains, whereas s2m2 strains have no cytotoxic activity, and s2m1 strains are rare [1]. Research showed that subjects infected with *vacA* s1 or m1 isolates have an increased risk of peptic ulcer and/or

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gastric cancer compared with those with s2 or m2 isolates [1–3]. Rhead *et al.* have recently described a novel determinant of VacA toxicity, the intermediate (i) region [4]. The duodenal ulcer promoting gene A (*dupA*) is another virulence determinant that has been shown to associate with duodenal ulcer (DU) in some countries. Also, the presence of *dupA* was found to be related to neutrophil infiltration and a high level of interleukin-8 (IL-8) production by epithelial cells [5,6]. Furthermore, *dupA* was previously classified into two main subtypes: the functional *dupA* with an extended open reading frame (ORF) within *jhp0917-19* (*dupA1*), and non-functional *dupA* with an early stop codon to truncate the ORF (*dupA2*) [5].

Since the discovery of *H. pylori*, the mainstream treatment of PUD has changed significantly [7]. The use of antibiotics to eradicate *H. pylori* infection is usually not chosen on the basis of routine susceptibility testing because of the difficulty of growing *H. pylori*. The eradication regimen should include a proton pump inhibitor and more than one antibiotic [7]. Historically, clarithromycin has been used as a first-line treatment for *H. pylori*. The eradication rate drops from 88% in the case of a clarithromycin-susceptible strain to <20% in the case of clarithromycin resistance [7]. With fluoroquinolones, which can be used as a second line of treatment, resistance was also shown to be correlated with treatment failure [8,9]. Because resistance rates vary according to the country and patient characteristics, the choice of antibiotics on the basis of susceptibility pattern in the region might be an effective strategy to improve *H. pylori* eradication [7]. Resistance to clarithromycin and fluoroquinolone is the result of mutations and these mutations are now well known [9–12]. Clarithromycin resistance is the result of point mutations in the *rrl* gene encoding the 23S rRNA. Three major mutations have been described: A2146C, A2146G and A2147G [10]. The resistance of *H. pylori* to quinolones results from point mutations in the *gyrA* gene encoding the A subunit of the DNA gyrase, mainly at codons 87 and 91 [9,11].

Helicobacter pylori virulence factors and their relationship with disease outcomes in Iraq were studied in this paper. In addition, the association of *dupA* polymorphisms with IL-8 secretion from gastric mucosa was studied. Also, molecular techniques were used to study the sensitivity pattern of *H. pylori* to clarithromycin and fluoroquinolone.

Materials and methods

Gastric biopsies

Gastric biopsies were obtained from 74 *H. pylori*+ patients from Iraq undergoing routine upper gastrointestinal endoscopy to investigate dyspepsia. Mean age ± standard deviation was 37 ± 13 years. Endoscopic diagnoses were: DU 19, gastric ulcer (GU) 10 and non-ulcer disease (NUD) 45. During gastroscopy, biopsy samples were taken and placed in 1 mL of isosensitest broth (Oxoid, Basingstoke, UK) containing 15% (volume/volume) glycerol and stored in liquid nitrogen. DNA was extracted directly from the biopsy specimens and used for PCR-based *H. pylori* typing.

The study protocol was approved by the Ethics and Research Committees of the individual hospitals and all patients gave informed consent to the study.

Antibiotic susceptibility

Helicobacter pylori antimicrobial susceptibility was investigated using mutational analysis to clarithromycin and fluoroquinolone using the GenoType HelicoDR kit (Hain Lifescience, Nehren, Germany) according to the manufacturer's instructions. To detect fluoroquinolone resistance, four gyr87 wild-type probes (gyr87WT1–gyr87WT4) and one mutant probe (gyr87MUT), and one gyr91 wild-type probe (gyr91WT1) and three mutant probes (gyr91MUT1–gyr91MUT3) were used to detect fluoroquinolone resistance at positions 87 and 91, respectively. To detect clarithromycin resistance, one wild-type probe (23SWT) and three mutant probes (23SMUT1–23SMUT3) were used with the presence of three controls: conjugate control (CC), amplification control (AC) and *H. pylori* (HP). When one of the wild-type probes stained positive together with the gyr91WT as well as 23SWT and no mutation band formed, the results were interpreted as susceptible to the respective antibiotic. The presence of a band at CC and AC meant that the CC and AC were in the right frame whereas a band at HP implied the presence of *H. pylori* according to the manufacturer's instruction.

Virulence factors genotyping

Thermal cycling for amplifying *cagA* was 95°C for 30 s, 50°C for 1 min, and 72°C for 2 min, for a total of 35 cycles. PCR amplification of *cagA* used previously described primers cag2 (5′-GGAACCCTAGTCGGTAATG-3′) and cag4 (5′-ATCTTTGAGCTTGTCTATCG-3′) to amplify about 500-bp product from the middle of *cagA* (PAI) [13]. For *vacA* signal and middle region, thermal cycle conditions were 30 s at 95°C, 60 s at 56°C and 90 s at 72°C, performed for 35 cycles. Primers used for the middle region were vag-F (5′-CAATCTGTCCAATCAAGCGAG-3′), and vag-R (5′-GCGTCTAAATAATTCCAAGG-3′). For the signal region, A3436 (5′-ATGGAAATACAACAAACACAC-3′) and C1226 (5′-CTGCTTGAATGCGCCAAAC-3′) primers were used [14]. The PCR conditions for *vacA* i region were as follows: the template mixtures were amplified 35 cycles with primers VacF1 (5′-GTTGGGATTGGGGGAATGCCG-3′) and C1R (5′-TTAATTTAACGCTGTTTGAAG-3′) for i1 region and VacF1

and C2R (5′-GATCAACGCTCTGATTTGA-3′) for i2 region, at 95°C for 30 s, 53°C for 1 min, and 72°C for 30 s [4]. The *dupA* was amplified using three sets of primers: DupA-WXF (5′-GATATACCATGGATGAGTTCYRTAYTAACAGAC) and DAR1 (5′-TTAAATACTCTTCCTTATAAGTTTCTTGG); JHP0919R2 (5′-GCCCACCAGTTGCAAAAACAAATGAAC) and DupA918F (5′-CCTATATCGCTAACGCGCTC); and dupA-F0 (5′-TGG CGT GTG GCA GTC TAA TGC) and dupA-R1 (5′-GCT CAA CAA AAT GCC CAC CAG TCG C) then, the amplified genes were sequenced on both strands using different primers (Table 1).

Mucosal IL-8 level

ELISA was used to measure the IL-8 levels in the antral biopsy specimens (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Briefly, samples were homogenized first and then aliquots of supernatant tissues were obtained by centrifugation. The total protein in the supernatants was measured by using the Bradford assay (Bio-Rad, Richmond, CA). ELISA sensitivities were approximately 5 pg/mL for IL-8.

Data analysis

Statistical analysis of data was performed by using stepwise logistic regression and the chi-squared test with significance set at a $p < 0.05$ using Minitab 15 software (Minitab Ltd., Coventry, UK).

Results

Association between virulence factors and clinical outcomes *cagA*, *vacA*, *vacAs*, *m*, combinations and *dupA* status

The prevalence of *cagA*+ strains in the study was 47.3% (35/74). Regarding the *vacA* genotypes, 70/74 (94.6%) of the studied strains typed as *vacAs1*. The *vacAm2* was found in 47 (63.51%) of our 74 samples. In addition, the *vacA i1* genotype was found in 42 of the 74 (56.76%). Regarding the combination of the s region, i region and m region of *vacA*, the prevalence of the genotype *vacA* s1i2m2 was highest (37.8%) (28/74), the prevalences of genotypes *vacA* s1i1m1 and s1i2m1 were 36.49% (27/74) and 20% (15/74), respectively. The genotype *vacA* s2i2m2 was found in only four patients, all of them with NUD (Table 2).

dupA was previously classified into two main groups: the common extended ORF within *jhp0917-19* (*dupA1*), and *dupA* with an early stop codon to truncate the ORF (*dupA2*) [15]. In our study the *dupA1* genotype was found in 12/74 (16.2%) and only 5/74 (6.7%) patients had the *dupA2* genotype.

The effect of the virulence factors on the disease outcome

DNA was extracted from gastric mucosa of 74 *H. pylori*-positive patients (19 DU, 10 GU and 45 NUD). The prevalence of *cagA*+ strains isolated from GU and DU patients was found to be significantly higher than those isolated from patients with NUD (90% and 57.9% versus 33.3%; p 0.01 for both).

All *H. pylori* strains isolated from patients with DU and GU had the *vacAs1* genotype, whereas 94.6% of the strains isolated from patients with NUD had the *vacAs1* genotype ($p > 0.05$). The *vacAm1* genotype was found to be more prevalent in strains isolated from DU (73.7%) and GU (70%) than those from NUD (14%) ($p < 0.01$). The prevalences of *vacAi1* strains isolated from DU and GU were 89.47% and 90%, respectively, and were significantly higher than that found in NUD (33.3%) (p 0.01 for both). The *vacA* s1i1m1 genotype was more prevalent in patients with DU (73.7%) and GU (70%) than those with NUD (13.3%) (p 0.01). The *vacA* s2i2m2 genotype was only found in patients with NUD (8.9%) (Table 2). Seventeen (22.9%) of our 74 strains typed as *dupA*-positive, among which 12 strains typed as *dupA1*. The prevalence of *dupA1* was higher in DU (36.8%) than in GU (10%) and NUD (8.9%) (p 0.01).

Multivariate analysis showed that *cagA*+/ *vacA* s1i1m2 strains were independently associated with the developing PUD with increased odds of developing PUD (p 0.03; OR = 2.1).

TABLE 1. Oligonucleotide primers used for DNA sequencing of *Helicobacter pylori* dupA

Primers	Sequences
DupA918F	CCTATATCGCTAACGCGCTC(9)
JHP0919R2	GCCCACCAGTTGCAAAAACAAATGAAC(9)
DAR1	TTAAATACTCTTCCTTATAAGTTTCTTGG(9)
DupA-WXF	GATATACCATGGATGAGTTCYRTAYTAACAGAC(9)
dupA-F0	TGG CGT GTG GCA GTC TAA TGC(26)
dupA-F1	GCA AAC GCT CAA ACT ATT GCC(26)
dupA-F2	ATG TTT CTT GGT TTA GAG GG(26)
dupA-F3	CAG AAC ACA AGC TTT AAA TGA ATT G(26)
dupA-F4	ATG AGT TCT ATA CTA ACA GAC TTTGAG CC(26)
dupA-F5	GGT TTC TAC TGA CAG AGC AC(26)
dupA-F6	GAT AAT TGG TAG CAC AGG AAG CGG(26)
dupA-F7	GGC TCT AGC GAA CAA GAT TTT AAT GAG(26)
dupA-R1	GCT CAA CAA AAT GCC CAC CAG TCG C(26)

TABLE 2. Association of vacA polymorphisms with clinical outcomes

Outcome (*n*)	s1i1m1, *n* (%)	s1i2m1, *n* (%)	s1i2m2, *n* (%)	s2i2m2, *n* (%)
DU (19)	14 (73.7)*	3 (15.8)	2 (10.5)	0 (0)
GU (10)	7 (70)*	3 (30)	0 (0)	0 (0)
NUD (45)	6 (13.3)	9 (20)	26 (57.8)	4 (8.9)
Total (74)	27 (36.4)	15 (20.2)	28 (37.8)	4 (5.34)

*Significant association.

The prevalence of clarithromycin and levofloxacin resistance and its association with virulence factors

Two mutations were associated with clarithromycin resistance: A2147G and A2146G. Levofloxacin resistance is associated with D91N mutation. The prevalence of these mutations was studied. Overall in 74 patients, 12/72 (16.2%) strains were clarithromycin resistant, of which ten carried the A2147G mutation (Table 3). Only three patients were levofloxacin resistant. The A2147G mutation was found in 8.1% in patients with NUD, 4.1% in patients with DU and 1.4% in patients with GU. The other mutation, A2146G, was found in only 2.7% of patients. 1.4% of patients with NUD and 1.4% of patients with DU tested positive for the presence of this mutation. The levofloxacin-resistance mutation (D91N) was found in three of the 74 patients (4.1%; one DU, one GU and one NUD).

A significant association was found between *dupA1* genotype and A2147G clarithromycin resistance mutation ($p < 0.01$) (Table 3). No significant association was found between A2146G or D91N and virulence factors.

The association between IL-8 and *dupA* status

To compare the levels of gastric IL-8 secretions, we classified our samples into three groups: *dupA*-negative, *dupA1* and *dupA2*. We found no significant difference between the levels of IL-8 secretion in gastric mucosa infected with *H. pylori dupA*-negative mucosa and *H. pylori dupA1*-positive strains (*dupA*-negative: mean ± median: 28 ± 26 versus 30 ± 27.1 for *dupA1*; p 0.6). Also, no significant difference was found between *H. pylori dupA*-negative mucosa and *H. pylori dupA2*-positive strains (28 ± 26 versus 35 ± 26; p 0.7) (Fig. 1). Also, we classified our samples into two groups: samples infected with *cagA*-positive strains and those infected with *cagA*-negative strains. It was found that the same levels of IL-8 were elicited in biopsies infected with *cagA*-positive strains and *cagA*-negative strains (*cagA*-negative: 29 ± 25 pg/mL; *cagA*-positive: 28.4 ± 27 pg/ml) (Fig. 2).

TABLE 3. The relation between antibiotic resistance and virulence factors

	Clarithromycin resistance		Levofluxacin resistance
	A2147G	A2146G	D91N
cagA	27%	0%	6%
dupA1	50%*	0%	0%
dupA2	0%	0%	0%
vacA s1	13%	3%	4%
vacA s2	25%	0%	0%
vacA m1	15%	0%	7%
vacA m2	13%	4%	2%
vacA i1	15%	0%	5%
vacA i2	12%	6%	3%
vacA s1m1	15%	0%	8%
vacA s1m2	12%	5%	2%
vacA s2m2	25%	0%	0%

*Significant association.

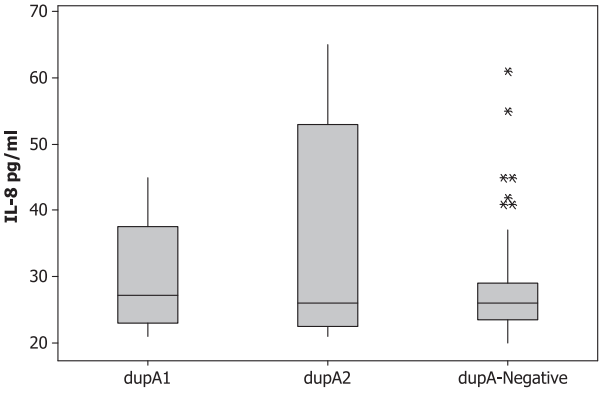

FIG. 1. Interleukin-8 levels in gastric biopsies from *cagA*-negative *Helicobacter pylori*-infected tissues, *dupA1 H. pylori*-infected tissues and *dupA2 H. pylori*-infected tissues. No significant differences were found; * indicates outlier.

Discussion

Helicobacter pylori infection is associated with peptic ulceration and gastric adenocarcinoma, the second highest cause of cancer deaths worldwide [7]. Clarithromycin and fluoroquinolone are two drugs used widely in the eradication of *H. pylori*. Resistance to these drugs is emerging and presents a challenge. Studies all over the world have reported resistance to clarithromycin and fluoroquinolone [16–18]. Proton pump inhibitor–clarithromycin-containing triple therapy without previous susceptibility testing should be abandoned when the clarithromycin-resistance rate in the region is between 15 and

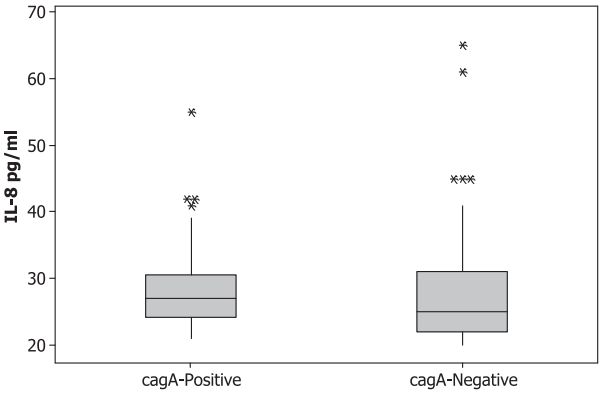

FIG. 2. Interleukin-8 levels in gastric biopsies from *cagA*-negative *Helicobacter pylori*-infected tissues and *cagA*-positive *H. pylori*-infected tissues. No significant differences were found; * indicates outlier.

20% [7]. No study has been conducted in Iraq to examine the resistance rate to these medications. It was found that 16.2% of the strains used in this study were clarithromycin resistant. These results need to be confirmed by culture and sensitivity and if true, then clarithromycin-containing triple therapy should not be considered for the eradication of *H. pylori* because of the high failure rate. In addition, only three (4%) patients were levofloxacin resistant. This is a low resistance rate in comparison to isolates from Belgium, France, Italy and Germany where higher resistance rates to ciprofloxacin or levofloxacin ranging between 16.8% and 23% [16,17] were found. Also, higher resistance rates (ciprofloxacin 33.8%; levofloxacin 21.5%) have been observed in Japan [18]. These differences in resistance rate could be attributed to geographical region and drug usage differences.

The study of *H. pylori* virulence factors in populations is important because they contribute to disease risk. For example, in Iran, where gastric cancer is common, more than 75% of *H. pylori* strains are *cagA* positive [2]. We looked within our populations for the associations between virulence factors and PUD. In agreement with previous reports from Iraq [19–21], a significant association between *cagA* status and PUD was observed. Also, research from a neighbouring country, Turkey, has shown results similar to those from Iraq [3]. Next, we examined the association between *vacA* polymorphisms and clinical outcomes. The *vacA* s1, m1 and s1m1 genotypes were significantly associated with PUD. This is in agreement with other reports from Iraq and other countries [2,22]. The *vacA* i region polymorphisms are a relatively a new virulence determinant and have not been examined thoroughly. It was previously shown that the *vacA* i1 genotype is associated with gastric cancer in Iran [4]. We studied the association between *vacA* i region polymorphisms and clinical outcomes. It was found that *vacA* i1 is significantly associated with GU and DU. A previous report from Iraq showed a significant relationship between *vacA* i1 and GU but not DU [2]. However, the sample size used in the previous study was small and more study was suggested to investigate such a relationship.

Multivariate analysis showed that *cagA*+/ *vacA* s1i1m2 strains were independently associated with the developing PUD. Other virulence factors, solely or in combination, could not show any independent association with clinical outcome. Therefore we suggest that single bacterial factors could not solely explain the outcomes of gastroduodenal diseases.

The *dupA* is a virulence factor that comprises both jhp0917 and jhp0918. Lu *et al.* found a significant relationship between *dupA* and DU, and the presence of *dupA* was related to neutrophil infiltration and a high level of IL-8 production by epithelial cells [6]. Since then, contradicting results reported about the relationships of *dupA* and clinical outcomes [5]. Then, *dupA* was classified into *dupA1* (functional) and *dupA2* (non-functional, including the originally described form, in which the the ORF was broken by a stop codon) [5]. Such a classification helped the explanation of contradicting results. In agreement with a previous report from Iraq, we found a significant relationship between *dupA1* and DU [15]. In addition, a previous report showed an association between *dupA* status and failure of *H. pylori* eradication [23]. No explanation was given in that report about how *dupA* could prevent the eradication of this bacteria. We found a novel association between *dupA1* and A2147G, a mutation responsible for clarithromycin resistance. Such an association is difficult to explain and warrants further study. However, this relationship might be because *dupA1* may increase the pro-inflammatory factors including reactive oxygen species and these factors may help to induce mutations in the bacteria. Further study is needed to confirm such a result and explore how these genotypes may have become associated.

Lu *et al.* found that the presence of *dupA* was related to neutrophil infiltration and a high level of IL-8 production [6]. We also previously reported that *dupA1* correlates with clinical outcome and gastric IL-8 levels in Iraqi *H. pylori*-infected samples [15]. However, such a relationship could not be obtained in this report. This contradiction is difficult to explain and warrants further studies.

To conclude, there is a high resistance rate to clarithromycin in our region. Such results need to be confirmed and, if true, it should be discussed whether clarithromycin should be dropped from the first-line eradication regimen. Univariate analysis showed that *cagA*, *vacA* s1, m1, and s1m1 genotypes were significantly associated with PUD. However, multivariate study showed that only *cagA*+/ *vacA* s1i1m2 combination is an independent marker for disease outcome. *dupA1* was shown to be associated with DU and clarithromycin resistance but not IL-8 secretion. The association between *dupA1* and clarithromycin may help to explain the previous report of the association between *dupA* and treatment failure. Further study is needed to explore the relationship between *dupA* polymorphisms and clinical outcomes and drug resistance.

Conflict of interest

The authors declare that there are no conflicts of interest.

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