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Research Article

INVESTIGATION OF *HELICOBACTER PYLORI* VIRULENCE GENOTYPE IN GASTRIC BIOPSIES BY POLYMERASE CHAIN REACTION

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ABSTRACT

Objectives: *Helicobacter pylori* infections have been associated with the genetic diversity of their virulence factors; the virulence genotypes are valuable as a molecular marker in the diagnosis of patients with bacterial infections. Our main objective was to analyze the frequency and allelic genotype of vacuolating cytotoxin A (*vacA*) and cytotoxin-associated gene A (*cagA*) as well as investigate other virulence genes like outer inflammatory protein A gene (*oipA*) and a gene contact with the epithelium (*iceA*) of *H. pylori*.

Methods: A total of 75 patients with gastritis and peptic ulcer diseases (PUDs) were selected to investigate *H. pylori* infections. 75 antrum biopsies were collected from these patients, and then genomic DNA was extracted from biopsies using the genomic DNA kit. Subsequently, the virulence genes of *H. pylori* were amplified using specific primers for *vacA*, *cagA*, *cagE*, *iceA,* and *oipA* by polymerase chain reaction (PCR).

Results: A high prevalence of genes *cagA* (28.6%), *vacAs1bm2 (*44.68%), *iceA2 (*30.6%), and *oipA* (42.9%) was found, while *vacA* s2m1 and *iceA1* genotypes was not found in our study. There was a significant correlation between the presence of *cagA* and *cagE* genotypes (p=0.02), suggesting that these two genes almost used together as a cagPAI integrity marker and the predominant *cagA* EPIYA motif was ABC (~650 bp) belong to Western *cagA* strains. The presence of the $cagA^+$ and $vacA^+$ genes was significantly associated with peptic ulceration ($p \leq 0.001$ at a level 0.01 and $p \leq 0.044$ at a level 0.05, respectively), whereas different *iceA2* genotype was no statistically significant with clinical outcome. Patients with PUD more likely to have an *oipA* gene (61.9%) than those with gastritis (38.1%), $p \leq 0.037$, also the presence *oipA* gene was statistically significant with presence *iceA2* and *cagA*, as well as the presence *iceA* gene was statistically significant with the presence *vacA*. There were 11 different genotype combinations and the most prevalent genotypes were $cagA^-/vacA^+/oipA^-/iceA2^-$ (31.9%), $cagA^-/vacA^+/oipA^+/iceA2^-$ (14.9%) followed $cagA^+/vacA^+/oipA^+/iceA2^+$ (10.6%), which associated with severe pathologies than the first and the second type.

Conclusion: Most *H. pylori* genotypes which associated with peptic ulcer and gastritis were moderate virulent strains, whereas the virulent strain which associated with peptic ulcer belong to Western *cagA* strains had *vacAs1bm2* genotype, *oipA* and *iceA2* genes that rarely induced gastric cancer in the middle region of Iraq.

Keywords: *Helicobacter pylori*, Gastric biopsies, Virulence genotyping, Cytotoxin-associated gene A, Vacuolating cytotoxin A, *oipA*, *iceA.*

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INTRODUCTION

Helicobacter pylori causes severe disease such as corpus gastritis, gastric atrophy, gastric ulcer, and increased risk of gastric cancer or gastric mucosa-associated lymphoid tissue lymphoma, in addition to developing a duodenal ulcer after gastric mucosa colonization [1]. The clinical outcome of this infection associated with many factors like host genetic factors, environmental factors and virulence factors of *H. pylori* which diverse according to geographical area and a vital role in bacterial pathogenicity. Some of these virulence factors are encoded by cytotoxin-associated gene A (*cagA*), *vacA*, *iceA*, and outer inflammatory protein A (*oipA*) genes [2,3].

Molecular methods are widely used to diagnose *H. pylori* infections as well as for analyses of virulence diversity and resistance patterns of these bacterial strains [4,5]. However, the high degree of genomic plasticity among strains of *H. pylori* caused the complexity of choosing the target genes. Even nucleotide sequences that are highly conserved in various strains of the pathogen, such as urease A (*ureA*), urease C (*glmM*) and *16S rRNA*, may fail to detect the bacteria [4,6]. Therefore, another virulence genes can be detected as *vacA*, *cagA* genes to confirm the presence of virulent *H. pylori* strains [3,7].

H. pylori produce large amounts of urease, an enzyme that hydrolyzes urea into ammonia and carbonates this mechanism to protect itself from acute acid shocks when it colonizes at gastric epithelial cells because of *H. pylori* is not an acidophil [8]. Urease enzyme encoded by *ure* operon that containing three clusters of genes, one of them is *ureA* which was used in bacterium molecular diagnosis. Furthermore, the bacterium has another gene (*glmM* or *ure*C) located upstream of the *ure* operon, but this gene does not participate in urease production, it encodes phosphoglucose amine mutase and it participates directly in cell wall synthesis. So both *ureA* and *ureC* considered "housekeeping" genes. However, they have been extensively used for confirming the presence of *H. pylori* [9,10].

Furthermore, *H. pylori* possess various virulence factors as the cytotoxin-associated gene A (*cagA*) and the vacuolating cytotoxin (*vacA*) that has been implicated in more severe responses within the host tissue. CagA is a cytotoxin that is translocated into host cells by the *cag* Type IV secretion system (*cag*-T4SS), encoded by several genes within the *cag* pathogenicity island (*cag*-PAI) [11]. CagA in either its phosphorylated form or its nonphosphorylated form can interact with at least 10 host cell components, resulting a complex assortment of cellular modifications. The level of CagA tyrosine phosphorylation in host cells is influenced by the number of EPIYA motifs within the protein that can undergo phosphorylation. *CagA* proteins containing higher numbers of EPIYA motifs exhibit enhanced binding to intracellular targets and enhanced activity within host cells, and strains have higher numbers of EPIYA motifs are related to greater gastric cancer

risk [11,12]. CagA associated with different changes to gastric tissue, including upregulation of proinflammatory cytokines, alteration of the actin cytoskeleton, disruption of metal homeostasis, and aberrant cell signaling [12]. VacA is a pore-forming toxin encoded by *vacA* gene and secreted by *H. pylori* that cause cell vacuolation, autophagy, inhibition of T-cell proliferation, and induction of programmed necrosis [7,12]. As *vacA* gene show polymorphism among strains since it has four regions: A signal sequence consisting of the allele s1, which has three subtypes: s1a, s1b, s1c and s2; an intermediate sequence; a deleted sequence (d); and a middle sequence that may have the m1 or m2 alleles [7,13,14]. The mosaic combination of s and m-region allelic types determines the particular cytotoxic and, consequently, the pathogenicity of the strain [7,14]. The most virulent strain is an s1m1 variant that produces a high level of cytotoxicity and associated with disease duodenal ulcer, the peptic ulcer disease (PUD) and gastric cancer as well as precancerous lesions and intestinal metaplasia, in addition to a wide variety of alterations in host cell biology [12,15].

The induced by contact with the epithelium A gene (*iceA*) has two main allelic variants, *iceA1,* and *iceA2*. The *iceA1* allele demonstrated sequence homology with a gene from *Neisseria lactamica*, nla IIIR, which encodes a cancer testis antigens-specific restriction endonuclease, but the similarity at the protein level is limited, due to frame shift mutations of *iceA1* in most *H. pylori* strains [16]. Shiota *et al.* reported that the meta-analysis confirmed there was a significant association between the presence of the *iceA1* allele and peptic ulcer, but there was inversely associated with the presence of *iceA2* and peptic ulcer, as well as *iceA* gene was not associated with gastric cancer. Furthermore, they reported that there was no correlation between the presence of *cagA* and *iceA1* allele [17]. The expression of *iceA1* was upregulated on contact between *H. pylori* and human epithelial cells, and the *iceA1* genotype was linked with enhanced mucosal interleukin (IL-8) expression and acute antral inflammation [17,18].

Outer inflammatory protein A encoded by the *oipA* (*hop*H) gene and this gene may be functional or nonfunctional (status "on" or "off") depending on a variable number of cytosine–thymine dinucleotide repeats in the 5' region of *oipA* [2,19,20]. The presence of a functional gene is significantly associated with the presence of duodenal ulcers, gastric cancer, and increased neutrophil infiltration. OipA expression is linked to increased IL-8 production *in vitro*; moreover, there is a strong link between the OipA "on"-status and *cagA* [19-21].

However, the main objective of researcher attention has more than one aspects including antimicrobial resistance properties of *H. pylori* and investigate new drug to treat infections [3,15,22,23], molecular diagnosis or molecular analysis to investigate its role in gastric cancer [2,7,15]. In Iraq, *H. pylori* infections were diagnosed according to classical methods such as enzyme-linked immunosorbent assay, rapid chromatography strip assay, and rapid urease test where were performed at hospitals and the histological identifications were performed at private laboratories. To develop and applied the molecular diagnosis methods in Iraqi Hospitals, the recent researches scope of this field to construct a molecular database for local microbial species like *H. pylori*. In our previous studies (2012), *H. pylori* was isolated from antrum biopsies of patients suffering from various gastroduodenal diseases and identified according to classical methods, microbial, immunological and biochemical characteristics, in addition, to detecting *cagA* and *vacA* genes by polymerase chain reaction (PCR) using specific primers. The results of classical diagnosis appeared that 85.3% of patients had an immune response (specific antibodies) against *H. pylori*, and the percentage of isolation was 41.3% of peptic biopsies and depending on the PCR results were expected that these isolates related western isolates, as well as the studies, deal with antimicrobial resistance properties of *H. pylori* their association with plasmid profiles [24,25]. Whereas in 2015 *H. pylori* was detected directly in peptic biopsies of patients with gastritis and peptic ulcer using specific primers for *ureC*, *ureA* and *16SrRNA* genes by PCR [24] and the results appeared 65.3% cases were positive for *H. pylori* in addition to it was more reliable and requiring short time for diagnosis in comparison with the classical identification methods. To determine the genotype of local *H. pylori* strains that contributed gastroduodenal disease in the middle region of Iraq, the main objective of this study to investigate allelic polymorphism of *vacA* gene as well as *cagA* integrity and detection other virulence genes, also detect the association of these genes with gastric disease's outcome. The next study determines the sequences of virulent genes to predicate the more specific primer to detect local *H. pylori* strains.

METHODS

Specimens sampling

This study was performed in Biotechnology and Genetic Engineering Laboratory at Biology Department, College of Science, Babylon University in Iraq. Endoscopy was performed by a gastroenterologist at esophagi-gastroduodenal scope (OGD) unit at Morgan Hospital in Babylon Province. During the endoscopy, three biopsies were taken from the antrum, from February to May 2015. One of the antrum biopsies was used for rapid urease test at the hospital; two biopsies were used for genomic DNA extraction. Antrum biopsies were taken from 75 patients with dyspeptic symptoms including 38 gastritis, 28 PUD, 3 nodular growth non-malignant, and 6 were apparently normal. They attended service arguing dyspepsia, reflux, dysphagia, weight loss, anemia, and other symptoms; all of them met the inclusion criteria who were selected and identified by a specialist physician. The antrum biopsies were placed in sterile normal saline, transported to the laboratory using the cooling box, then frozen at −24°C until processing.

Genomic DNA extraction and purification

The frozen biopsy specimens were thawed and crushed and genomic DNA was directly extracted and purified using FAVORGEN tissue genomic DNA extraction mini kit, according to the manufacturer's instructions. DNA quantity and quality were determined using an ultraviolet-visible Spectrophotometer-NanoDrop instrument (OPTIZEN POP - Korea). The concentration of DNA was measured at 260 nm which ranged 0.1-1.0 ng/ml and the DNA purity ranged between 1.7 and 2.0.

PCR amplification and electrophoresis

For *H. pylori* genotyping, the target DNA regions were amplified using specific primers were mentioned in Table 1. The reaction mixtures were prepared using a PCR Master Mix (Promega-USA) according to kit instructions. Each PCR reaction was carried out in a final volume of 25 µl under different conditions were displayed in Table 1. We used a TRIO Thermal Cycler (Biometra-Germany) for gDNA amplification. PCR products were migrated and visualized in 1.5% (w/v) agarose gel electrophoresis in 1X of TAE buffer (Tris Acetate-Ethylenediaminetetraacetic acid buffer) composing from 0.040 mol Tris-acetate and 0.001 mol EDTA per L, pH 8.0±0.2., at 6 V/cm for 1 hr. Gels were stained with ethidium bromide solution (5 µg/ml) [26,27] using horizontal electrophoresis (Cleaver Scientific, UK) and Gel Documentation System (UVsolo touch, Biometra-Germany).

Data analysis

Data were analyzed using SPSS version 22 software Fisher's exact and Chi-square tests were used for analysis of cross table data, with a significant $p<0.05$ and correlation one-tail between genes and clinical outcome, with a significant $p<0.05$ and <0.01. Genotypes with mixed status for *vacA* were excluded from the calculations of association.

RESULTS AND DISCUSSION

A total of 75 patients included in this study and have presented functional dyspepsia and/or chronic gastritis without atrophy or intestinal metaplasia. In the previous investigation, the presence

Table 1: Primer and PCR conditions

Region	Primer	Sequence of primer 5'-3'	Size of PCR product (bp)	Initial denaturation °C (minutes)	Denaturation °C (seconds)	Annealing °C (seconds)	Extension °C (seconds)	Cycles	Final extension °C (minutes)	References
s1/s2	VA1-F VA1-R	ATGGAAATACAACAAACACAC CTGCTTGAATGCGCCAAAC	259/286	94 (10)	94 (60)	53 (60)	72 (60)	35	72 (5)	[25,28]
s1b	SS3-F/VA1-R	AGCGCCATACCGCAAGAG	187	94 (10)	94 (60)	53 (60)	72 (60)	35	72 (5)	[25,28]
s1a	SS1-F/VA1-R	GTCAGCATCACACCGCAAC	190							
s2	SS2-F/VA1-R	GCTAACACGCCAAATGATGC	199							
m1/m2	HPMGF HPMGR	CACAGCCACTTTCAATAACGA CGTCAAAATAATTCCAAGGG	401/476	94 (9)	94 (30)	50 (45)	72 (45)	40	72 (5)	[29]
m1	VA3-F VA3-R	GGTCAAAATGCGGTCATGG CCATTGGTACCTGTAGAAAC	290	94 (5)	94 (30)	52 (30)	72 (30)	27	72 (7)	[25,28]
m2	VA4-F VA4-R	GGAGCCCCAGGAAACATTG CATAACTAGCGCCTTGCAC	352	94 (5)	94 (30)	52 (30)	72 (30)	27	72 (7)	[28]
m1/m2	MF1 MR1	GTGGATGCTCATACAGCTWA RTGAGCTTGTTGATATTGAC	107/182	94 (9)	94 (30)	50 (45)	72 (45)	40	72 (5)	[29]
cagA	CAGAF CAGAR	GATAACAGCCAAGCTTTTGAGG CTGCAAAAGATTGTTTGGCAGA	349	95 (60)	95 (60)	55 (60)	72 (60)	35	72 (5)	[28]
cagE	M13F cagER	TGTAAAACGACGGCCAGTGGG GGAATAGGTTGTTTGGT GGATCACCCCATCATCTAAAAA	380	95 (10)	95 (30)	55 (60)	72 (45)	35	72 (10)	[30]
Cag-PAI empty site	M13 F T7 R	TGTAAAACGACGGCCAGTACA TTTTGGCTAAATAAACRCTG TAATACGACTCACTATAGGGT CATGCGAGCGGCGATGTG	380	95 (10)	95 (30)	50 (60)	72 (45)	35	72 (10)	[30]
iceA1	iceA1-F iceA1-R	GTGTTTTTAACCAAAGTATC CTATAGCCASTYTCTTTGCA	247	94 (10)	95 (60)	56 (60)	72 (60)	35	72 (10)	[28]
iceA2	iceA2-F iceA2-R	GTTGGGTATATCACAATTTAT TTRCCCTATTTTCTAGTAGGT	229/334	94 (10)	95 (60)	56 (60)	72 (60)	35	72 (10)	[28]
oipA	HPO638F HPO638R	GTTTTTGATGCATGGGATTT GTGCATCTCTTATGGCTTT	401	94 (10)	94 (60)	56 (60)	72 (60)	35	72 (10)	[28]

PCR: Polymerase chain reaction

of *H. pylori* infections was confirmed by PCR analysis using specific primers for *16SrRNA*, *ureC,* and *ureA* genes. The identification results showed 49 (65.3%) out of 75 biopsies gave positive results for at least two genetic markers including gastritis (55.26%) and PUD (100%) infections, but not the others [28]. Therefore, the total specimens harboring *H. pylori* which enrolled in this study were 49 cases.

The gDNA of the studied cases were analyzed for *vacA* and *iceA* allelic variant detection and *cagA* and *oipA* genotyping using specific primers by PCR technique.

vacA genotyping

The results of *vacA* genotyping revealed that 42 (85.71%) out of 49 cases were harboring *vacA* gene, which composed of single and middle regions including s1m1, s1m2 or s2m2 allelotypes, but the allelotype s2m1 were not found. Two cases of them had two types of s-region (s1 and s2) as well as m-region (m1 and m2), so they were excluded from the calculations of association, that may indicate these patients had multiple infections by two different *H. pylori* strains. The rest cases (12.24%) gave a negative result for the presence of *vacA* gene. All the PCR results were repeated more than three times using the primer pairs to amplify *vacA* s-region, including VA1F/VA1R and SS2-F/VA1R which resulted 259/286 bp for s1/s2 and 199 bp for s2 allelotypes, as well as *vacA* m-region was amplified using primer pairs VA3 (290 pb) for m1 type and VA4 (352 pb) for m2 type or HPMG (401/476 pb) for m1/m2 types (Fig. 1). Our finding of s1 allelotype was distributed as 89.3% and 42.1% for individuals with PUD and gastritis, respectively. These results confirmed previous studies [26,27]. Noticeable samples in our study with a nodular growth have no detected any genes for *H. pylori*. For s2 allelotype, the distribution was 10.7% for PUD and 21.1% for gastritis; in the current study, there is no significant between the manifestation of disease and single region of *vacA* gene.

The positive cases (40) had s1 type (70.22%) or s2 type (14.89%) and m1 allele (12.77%) or m2 allele (72.34%). The relationship between *vacA* allelotypes and clinical outcome revealed that s1 type was dominated by individuals with PUD (89.29%) and followed gastritis (42.1%), also m2 type was dominated by individuals with PUD (85.71%) and followed gastritis (52.62%). As a result of the allelotype *vacAs1m2* combined with the PUD and gastritis infections as a percentage 75% and 31.57%, respectively (Table 2).

The s1 allele of *vacA* gene consists from three subtypes are s1a, s1b, and s1c, in our study only s1a and s1b subtypes were investigated, but not an s1c subtype because of it is exclusively found in East-Asian isolates [31]. The positive results of s1 type within 32 cases were consisting from s1a (18.75%), s1b (75%) as shown in Table 2, whereas two cases (6.25%) were negative for both s1a and s1b subtypes; that may be indicated they had the third subtype which was not investigated in the present study that it may be classified into s1c. We think our study of allelotyping of *vacA* gene investigation is the first study in the middle region of Iraq, which found the allelotype *vacAs1bm2* is the predominant allele (44.68%) related to gastritis and peptic ulcer. The results may be indicated, these isolates produced moderate vacuolating toxin and had moderately virulent in gastritis infection and peptic ulceration. However, the presence *vacA* gene within these isolates was increasing the pathogenicity and it had a statistically positive correlation with the disease (p=0.01 at a level 0.05).

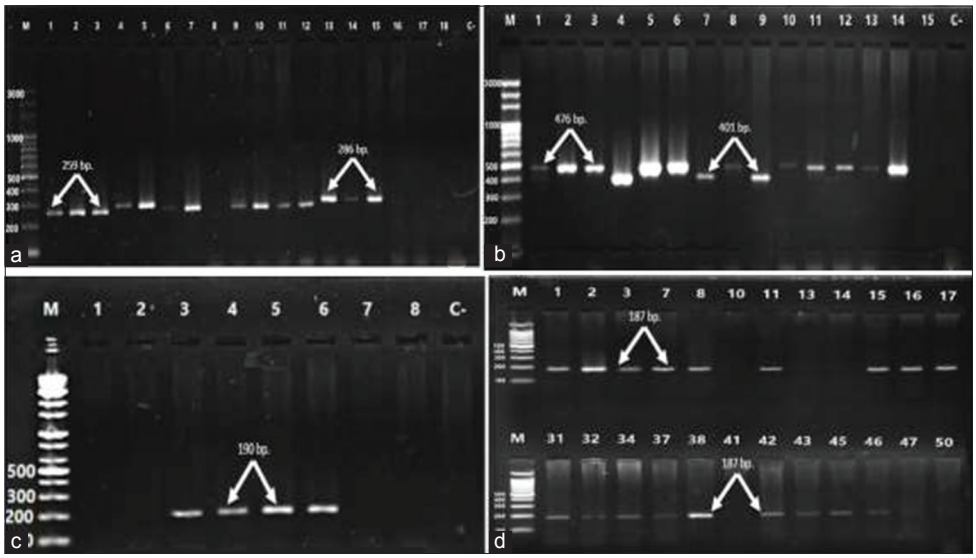

Fig. 1: Gel electrophoresis of polymerase chain reaction products of vacuolating cytotoxin A genotyping

Table 2: Relationship between *vacA* genotypes and manifestation of diseases

Genotype	PUD N=28 (%)	Gastritis N=19 (%)	Total N=47 (%)	p value (0.05)
vacAs2m2	3 (10.71)	4 (21.05)	7 (14.89)	0.339
vacAs1m1	4 (14.29)	2 (10.53)	6 (12.77)	0.712
vacAs1m2	21 (75)	6 (31.57)	27 (57.45)	0.008
Total	28 (100)	12 (63.16)	40 (85.11)	0.01*
s1-region subtype				
vacAs1am1	2 (7.14)	0	2 (4.26)	0.243
vacAs1am2	3 (10.71)	1 (5.26)	4 (8.51)	0.522
vacAs1bm1	2 (7.14)	1 (5.26)	3 (6.38)	0.801
vacAs1bm2	16 (57.14)	5 (26.32)	21 (44.68)	0.088
Total	23 (82.13)	7 (36.84)	30 (63.83)	

**vacA* was statistically positively correlated with peptic ulcer and gastritis diseases. (P=0. 01, significance level=0. 05) PUD: Peptic ulcer disease, VacA: Vacuolating cytotoxin A

1.5% agarose gel at 6 V/cm for 1 hr; C: Negative control (*Escherichia coli* MM294), M: 100 bp DNA marker.

A. PCR product of s1 and s2 types 259 bp and 286 bp, respectively; Lane 1 - 3, 6, 7, 10-12: Positive for s1 type, Lane 4, 5, 13-15: Positive for s2 type, mixed strain lane 9, other lanes were negative
B. PCR product of m1 and m2 type 401 bp and 476 bp, respectively; Lane 4, 7, 9: Positive for m1 type, Lane 2, 3, 5, 6, 8, 10-14: Show positive results of m2 type, Lane 1: Positive to both m1 and m2, Lane 15 was negative
C. PCR product of s1a subtype 190 bp; Lane 3, 4, 5, 6: Positive for s1a subtype, others were negative
D. PCR product of s1bsuballelic 187 bp; Lane 1-3, 7, 8, 11, 15-17, 31, 32, 34, 37, 38, 42, 43, 45, 46: Shows positive results for s1b subtype, other lanes were negative.

In comparison with the other studies that performed in the middle east region like Turkey and Egypt, which found *vacAs1a* allele is dominant (70.1% and 72.5%, respectively) than *vacAs1b* allele (2.8% and 7.2%, respectively) and 27.1% and 21.7%, respectively, as vacA s2 [32,33]. The allelotype s1a is more active than s1b [29], and with other *vacA* genotypes, the combination is associated with greater pathogenicity and virulence related to gastric cancer and peptic ulceration [34,35]. Thus, the results of vacA s-region variation indicate that there is geographical variation within *H. pylori* strains. In comparison with other geographical regions the s1a alleles are predominantly in strains from northern and eastern Europe, whereas the s1b allelic types are common in such regions as Central and South America, Spain, Portugal, and South Africa. The s1b subtypes are rare in other regions and less virulent than other s1 subtypes [36,37]. The strains in the present study belong to western type, as studies indicated s1b subtype is rarely found in East-Asian countries [29,32,38]. Our results revealed m2 as the main allele that similar to studies from Iraq, Turkey, Iran, Saudi Arabia, and the middle east region characterized m2 as the main allele [14,32,33,39-42]. In another hand, some studies reported that the predominant *vacA* m subtype was m2 and no association or no role between *vacA* m genotypes and the development of peptic ulcer [2,32,33,42,43], whereas other studies had been found that *vacA* m1 strains are associated with high levels of inflammation in the gastric mucosa and increased the risk of gastric atrophy and carcinoma [2,44]. However, the results were different from country to others, but we consistent with different studies those of Middle Eastern countries, in which the *vacA* s1 and m2 subtypes have been predominant subtypes [32,42,45,46].

Essentially, all strains possess the *vac*A gene, but not all secrete functional active VacA proteins (biologically active toxin) due to the variation of gene structure [2,7]. Reports in different geographic regions (Middle East, Africa, and Western) were demonstrated that individuals infected with *H. pylori* strains harboring *vac*A s1 allele have an increased risk of peptic ulcer or gastric cancer compared with individuals infected with strains had *vac*A s2 type [2,41,47,48]. Whereas *H. pylori* with s2 having minor cytotoxic activity than s1 [2,39,48]. Moreover, *H. pylori* strains have diverse combinations of *vacA* s and m regions and each strain possesses a unique pattern according to these combinations level of virulence of *H. pylori* is evaluated. The differences in allelotyping of *vacA* gene allow for differences in vacuolating activities between strains, and encoding proteins with different cytotoxicity, so *vac*A s1/m1 strains produce proteins with the high level of cytotoxicity and induce high vacuolation in gastric mucosa, while s1/m2 strains produce proteins have moderate level of action, and there is typically no vacuolating activity in s2/m2 strains [2,29]. Furthermore, heterogeneity among *vacA* alleles may be an important factor in understanding variations in clinical manifestations among *H. pylori*-infected subjects.

***cagA* genotyping**

cagA gene is encoded *cagA* protein, where CagA is another toxin that enables the bacteria to bring damage to the host cell. *H. pylori* strains possess *cagA* gene are more virulent and associated with increasing risk for developed PUD, atrophic gastritis, and gastric cancer, and there is a direct link between the presence of CagA and an increased cancer risk [7,42]. The results revealed 14 from 49 samples positives for *cagA* gene by using cagA primers with product size 349 bp (Fig. 2a), the percentage of *cagA* in our study was 28.6%, as lower as percent how reported by Hussein *et al*. [49] were 71% of study samples where were performed in Iraq at the different region, Kurdistan. Thus, suggesting there is wide variation in Iraqi strains those from north to those from the middle and south region. Furthermore, our results were different with the other countries were found the prevalence for *cagA* status in middle eastern countries like Jordan 26.4%, Saudi Arabia 52%, Kuwait 41%, Iran 76%, Turkey 78%, Egypt 35.7% [32,42,50]. Whereas East Asian, Western European, Latin American, Tunisian, and Lebanese strains have been revealed to have *cagA* positivity of nearly 100%, 80%, 70%, 61.6%, and 37.5%, respectively [30,51-53].

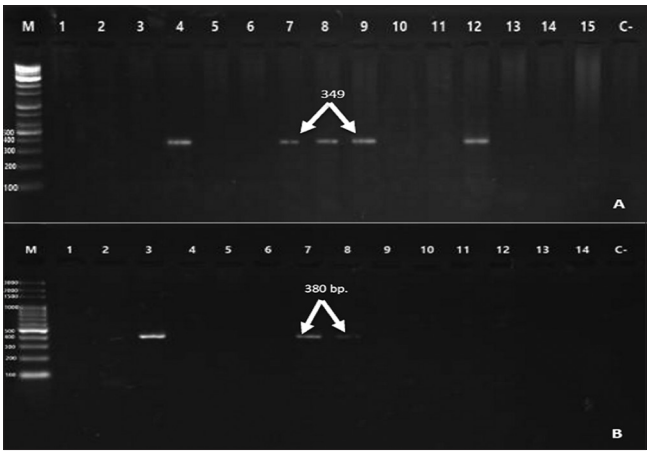

Fig. 2: Gel electrophoresis of polymerase chain reaction product of cytotoxin-associated geneA

1.5% agarose gel at 6 V/cm for 1 hr.

A. Gel electrophoresis of PCR product of *cagA* gene 349 bp
   Lane 4, 7, 8, 9, 12: Positive for *cagA* gene, Lane 1-3, 5, 6, 10, 11, 13-15 negative results, C: Negative control (*E. coli* MM294, M: 100 bp DNA marker)
B. Gel electrophoresis of PCR product of *cagE* gene 380 bp.
   Lane 3, 7, 8: Positive for *cagE* gene, Lane 1, 2, 4, 5, 6, 9, 10-14 negative results, C: Negative control (without DNA), M: 100 bp DNA marker.

However, the results show there was a significant relationship between the presence of *cagA* and peptic ulceration p≤0.001; the significant association confirmed by previous reports from Iraq and Turkey [49,50] but the presence of *cagA* was higher than our finding this may be due to a regional area of research. Other middle eastern countries had been no significant between *cagA* status and incidence of diseases such as Turkey, Egypt, and Iran [30,32,39,42].

Table 3: *cagA* status with *vacA* polymorphisms of *H. pylori*

Combination *vacA* gene	*cagA*$^+$ N (%)	*cagA*$^-$ N (%)	Total N (%)
*vacA*s1m1	4 (28.6)*	2 (6.1)	6 (12.77)
*vacA*s1m2	7 (50)	20 (63.6)	27 (59.57)
*vacA*s2m2	0	7 (21.2)	7 (14.89)
vacA$^-$	3 (21.4)	4 (9.1)	7 (12.7)
Total	14 (29.8)	33 (70.2)	47 (100)
*vacA*s1 subellelic			
s1am1	2 (14.3)	0	2 (4.1)
s1am2	0	4 (12.12)	4 (8.2)
s1bm1	2 (14.3)	1 (3.03)	3 (6.1)
s1bm2	7 (50)	15 (45.45)	22 (42.9)
Total	11 (78.6)	20 (60.6)	31 (87.23)

*Statistically significant p=0.04 (significance level=0. 05). *cagA*$^+$: The isolates harboring the *cagA* gene, *cagA*$^-$: The gene not found, *H. pylori: Helicobacter pylori, VacA*: Vacuolating cytotoxin A

Among *cagA* positive strains, the most predominant *vacA* patterns in our study as shown in Table 3 were s1bm2 (50%), interestingly, *cagA* positive strains statistically correlated with s1m1 pattern p=0.04, odds ratio 6.60 (confidence interval 95% 1.05; 41.51), but not to others, p>0.05 for all others patterns. A study by Honarmand-Jahromy *et al*. [39] was performed in Tehran, Iran, whom found the *vacA s1m2cagA*$^+$ genotype was the most prevalent within the three disease groups including chronic gastritis, gastric ulcer and duodenal ulcer patients. In addition to the *vacAs1m2* genotype was predominant with 56.2% with a similar occurrence in all diagnoses, while vacA s1m1 most prevalent within duodenal ulcer patients (33.9%). Also, they found the genotype of vacA s2m2 occurred in 15% of isolates and was more common in chronic gastritis patients (21.2%); vacA s2m1 were the least common genotype.

cag-PAI is a major virulence factor associated with several gastroduodenal pathologies, to test the integrity *cag* pathogenicity island, we had test presence of the *cagE* gene. This gene belongs to the *cag* pathogenicity island and locates in the upstream region of the island, *cagE* encodes a protein that caused induction of IL-8, and also this protein is a part of *H. pylori cag* T4SS apparatus [11,54]. As reports suggested that this gene is a more accurate marker of an intact pathogenicity island and can be used as a *cag*-PAI marker with *cagA* [55]. The results appeared 4 (28.6%) out of 14 (positive *cagA*) samples have *cagE* (Fig. 2b) using specific primer for this region, amplification of *cagE* gene indicates these samples possess complete *cag* PAI, others samples that not yield, consider contain modified *cag*-PAI as well as might be lack *cagE* genotype. Moreover, another test was done by PCR assay using specific primer for the *cag* empty site to assess or confirm the loss of *cag*-PAI, the investigated amplicon for the empty site is about 380 bp, the results revealed six samples of not *cagA* positive have been lost *cag* PAI by giving 380 bp size amplicon. Suggesting these samples infected with *H. pylori* strains undergo deletion to *cag* PAI and it's completely absent, among others *cagA* negative did not give any amplicon, so it still contains a partial *cag* PAI. However, the 14 samples that possessed *cagA* positive strains did not yield any empty site amplicon of the expected size, thus the result confirms those samples have *cagA* genotype and as a consequence, *cag* PAI was existing.

Jones *et al.* [56] reported that the presence of the *cagA* gene did not strictly correlate with expression and delivery of CagA in all noncancerous strains. This supports the idea of presence modification in *cag* PAI or lack *cagE* genotype as a protein of this gene is a part of *H. pylori cag* T4SS apparatus and mediate transport *cagA* protein to host cells [11,54]. Furthermore, Monstein *et al*. [57] suggesting the presence of a deviating *cag*-PAI. While Hussein *et al.* [49] found *cagE* in all *cagA*$^+$ strains this contrary to our result.

The statistical analysis reveals a significant correlation between the presence of *cagA* and *cagE* genotypes (p=0.02), suggesting that these two genes almost used together as a *cag* PAI integrity marker. Also, the presence of *cagA* and *cagE* increased the accuracy [54].

Also, the polymorphisms of *cagA* gene variable 3' end investigated by PCR, 14 samples with positive *cagA* gene had subjected to PCR using cagA-EPIYA primers to amplified *cagA* EPIYA motif region as previously described by Monstein *et al*. [57]. The amplicons ranged in size between ~600 and ~900 bp using single pair primer to produce varying PCR amplicons size depending on number of *cagA* EPIYA motifs. The results appeared the presence of variable amplicons in different biopsies, where 5 samples give PCR product of ~650 bp; we expected the type of EPIYA in these samples is ABC and two samples give amplicon ~580 bp probably EPIYA type here AB or BC, this primer pair enable to detect EPIYA types based on size, in which EPIYA-ABC give ~650 bp EPIYA-C segment may multiplies variable, mostly from one time to three times, and give larger product size in EPIYA-ABCC or ABCCC ~850-900 bp among different Western CagA species, in our results not revealed predicting size for EPIYA-ABCC or ABCCC. While other *cagA* positive samples, we did not detect any EPIYA (From 7 biopsy DNA samples, no *cagA* EPIYA motif amplicons could be generated). In conclusion, the predominant *cagA* EPIYA motif was ABC with the size of ~650 bp and belongs to Western *cagA* strains, as EPIYA-C is predominantly found in strains from Western countries [7]. There are many evidence indicate that the risk of gastric cancer or premalignant lesions is higher in persons infected with cagA positive *H. pylori* strains than in persons infected with cagA negative strains [11,12]. The increased risk of gastric cancer observed with cagA-positive strains (which often contain the entire cag PAI) are attributed to the cellular effects of CagA, combined with an enhanced gastric mucosal inflammatory response [12].

***iceA* genotyping**

This gene related to some gastrointestinal disease. The *iceA* gene contains two allelic variants *iceA1* and *iceA2* [45,58]. Analysis of *iceA* gene based on PCR assay using primers specific to sequence region of *iceA* gene, which give products of 247 bp and/or 229/334 bp for *iceA1* and *iceA2* respectively, as shown in Fig. 3. The primers yield a fragment of 229 or 334 bp depending on the presence of a repetitive sequence of 105 nucleotides codifying for 35 amino acids in some *iceA2* alleles.

Overall, *iceA1* was not found in the present study of all 49 isolates and *iceA2* was found in 16 strains 34% and it was distributed between the PUD and gastritis at the percentage 28.6% and 42.1%, respectively, this revealed a new picture for *iceA* genotyping, the absence of *iceA1* may reflect a greater geographic divergence among strains in the present study compared with other studies that *H. pylori* genotypes are not uniformly distributed worldwide. As previously reported that the *iceA1* and the *iceA2* genotypes were, respectively, isolated in 60.2% and in 16% of strains [30]. The *iceA1* genotype associated with peptic ulceration in patients [2,16,30,58]. In our study, *iceA2* allele was considered predominant and there was no association between *iceA2* and *cagA* status. While Ozbey and Aygun [58] found a significant association between *iceA2* and the presence of *cagA*. The function of *iceA2* is unknown. In this study, combination *vacA/iceA2* and *cagA/iceA2* analysis demonstrated that the *vacA/iceA2* and *cagA/iceA2* were more prevalent in PUD patients (28.6% and 17.9%, respectively) than in patients with gastritis alone (21.1% and 10.5%, respectively). Whereas combination *vacAs1/iceA2* analysis demonstrated that the *vacAs1/iceA2* was relatively more prevalent in PUD patients (17.9%) than in patients with gastritis alone (15.8 %), may be due to there is no association between the size of the *iceA2* amplicon and diseases. The numbers in this study were relatively small which limited statistical analysis. These results were different with Kidd *et al.* [59] results who they found combination vacAs1/iceA analysis demonstrated that the vacAs1/iceA2 was more prevalent in PUD patients (53%) than in patients with gastritis alone (23%), whom they found an amplicon size of this gene related to discriminate between the PUD and gastritis alone because of their finding about the presence a significant relationship between the cassette structure of *iceA2* and expression *in vivo*; the induction of *iceA* may also contribute to disease outcome.

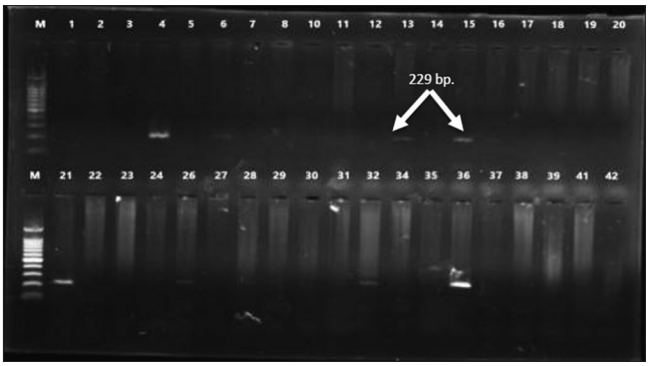

Fig. 3: Gel electrophoresis of polymerase chain reaction product of *iceA2* allele 229/334 bp

1.5% agarose gel at 6 V/cm for 1 h. Lane 4, 6, 13, 15, 21, 26, 32, and 36 were positive for *iceA2* allele, all other lanes were negative, M: 100-bp DNA marker.

oipA genotyping

The 5' region of the *oipA* gene was amplified using primers (HPO638F, HPO638R) to yield 401 bp PCR product (Fig. 4). The *oipA* genotype was detected in 21 (42.9%) out of 49 strains, and the others were *oipA* negative; this result nearly consistent with the results of Yamaoka [60] who found the *oipA* gene in studied *H. pylori* isolates with the percentage 45.9% of strains, while it was less than the finding (90.8% of strains) that documented by Ben Mansour *et al*. [30].

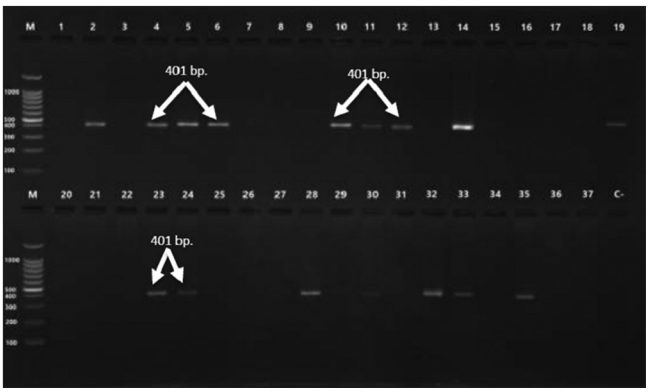

Fig. 4: Gel electrophoresis of polymerase chain reaction product of outer inflammatory protein A gene 401 bp

About 1.5% agarose gel at 6 V/cm for 1 hr. Lane 2, 4-6, 10-12, 14, 19, 23, 24, 28, 30, 32, 33, and 35 were positive for *oipA* gene, Lane 1, 3, 7-9, 13, 15-18, 20-22, 25-27, 29, 31, 34, 36, and 37 show negative results, C: Negative control (*E. coli* MM294), M: 100-bp DNA marker.

The *oipA* (HpO638) gene encodes an OipA, that associated with increased secretion of IL-8 [2,3,13,19,61], our investigation reveals that patients with PUD more likely to have an *oipA* gene than those with gastritis with the percentage 61.9% and 38.1%, respectively, with statistical significance p≤0.037. Souod *et al*. [14] showed that there was no association between this gene and gastric disorders also they found in another study that the *oipA* gene was found in 88 (71.54%) samples and statistically there was no association between this gene and gastric disorders [31].

Combined _cagA_, _vacA_, _iceA_ and _oipA_ genotypes

In our study, first, we have studied the relationship between virulence genes (*vacA, cagA, iceA,* and *oipA*) and the different gastroduodenal diseases, between the two groups (patients with peptic ulceration/patients with gastritis) $vacA^+$ negatively correlated with gastritis, and positively correlated with PUD (p≤0.001 at a level 0.01), also $cagA^+$ negatively correlate with gastritis and positively correlated with PUD (significance 0.044 at the level 0.05). The combination of the distinct *vacA, cagA, iceA,* and *oipA* genotypes illustrated the mosaic composition of the *H. pylori* genome. Based on analysis of the *cagA* gene (positive and negative), the *vacA* s-region (s1 and s2), the *iceA2* allelic type and the *oipA* gene (positive and negative), 11 different genotype combinations were recognized as shown in Table 4. The most prevalent genotypes were $cagA^-/vacA^+/oipA^-/iceA2^-$ (31.9%), $cagA^-/vacA^+/oipA^+/iceA2^-$ (14.9%) followed by $cagA^+/vacA^+/oipA^+/iceA2^+$ (10.6%), which associated with severe pathologies than the first and the second type. The first type was lower virulent than the second type as a result in most *H. pylori* strains that had been caused gastroduodenal diseases in middle Iraq are low virulence.

The overall results, we have examined the prevalence of *vacA, cagA, iceA,* and *oipA* genotypes of *H. pylori* strains clinically isolated in Iraq. No significant correlation was found between the expression of *cagA* and *iceA2* genes and the two groups of study patients, but the difference was statistically significant between the *vacA* and *oipA* genes. Also, the results revealed that the presence of *oipA* gene was correlated positively with the presence *iceA2* gene (p≤0. 024) and $cagA^+$ (p≤0.006 at a level 0.01), whereas *iceA2* positively correlated with *vacA* at (p≤0.006 at level 0.01).

Our different from Ben Mansour *et al.* [30] finding how reported the prevalence of the *vacA*, *cagA, iceA* and *oipA* genotypes of *H. pylori* strains clinically isolated from peptic ulcer and gastritis in Tunisia which typed as $vacA\ s2/cagA^+/iceA^+/oipA^+$ were more prevalent than those typed as $vacA$ s1/$cagA^+/iceA^+/oipA^+$ and which are related to severe pathologies, and no significant correlation was found between the expression of *cagA* and *iceA* genes and the two groups of studied patients, but the difference was statistically significant with the *vacA* and *oipA* genes. Moreover, they suggested that *vacA* gene possibly works as an immune modulator changing the immune response to the immunogenic *cagA*. The cytoskeleton of gastric epithelial cells is disorganized by *cagA*, and *vacA*, which leads to amplified cell spreading and growth. When *cagA* and *vacA* are combined, they can reduce the effect of each protein alone, probably leading to much more survival of infected host cells. This, possibly, occurs by ending *vacA* induced apoptosis or by inhibiting the *vacA* induced autophagy pathway by *cagA* [30].

Table 4: The genotype of _H. pylori_ and their combination with PUDs and gastritis

***H. pylori* genotype**	**Molecular identification[a]**	**Disease**		
	ureC/16SrRNA/ureA	**PUD N=28 (%)**	**Gastritis N=19 (%)**	**Total N=47 (%)**
$cagA^+, vacA^+$				
$cagA^+/vacA^+/oipA^+/iceA2^+$	+++	5 (17.9)	0	5 (10.6)
$cagA^+/vacA^+/oipA^+/iceA2^-$	+++	3 (10.7)	0	3 (6.4)
$cagA^+/vacA^+/oipA^-/iceA2^-$	−++	3 (10.7)	0	3 (6.4)
$cagA^+, vacA^-$				
$cagA^+/vacA^-/oipA^-/iceA2^+$	+++	0	2 (10.5)	2 (4.2)
$cagA^+/vacA^-/oipA^-/iceA2^-$	−++	0	1 (5.3)	1 (2.1)
$cagA^-, vacA^+$				
$cagA^-/vacA^+/oipA^+/iceA2^+$	−++	3 (10.7)	2 (10.5)	5 (10.6)
$cagA^-/vacA^+/oipA+/iceA2^-$	−++	2 (7.1)	5 (26.3)	7 (14.9)
$cagA^-/vacA^+/oipA^-/iceA2^+$	+++	0	2 (10.5)	2 (4.2)
$cagA^-/vacA^+/oipA^-/iceA2^-$	−++	12 (42.9)	3 (15.8)	15 (31.9)
$cagA^-, vacA^-$				
$cagA^-/vacA^-/oipA^-/iceA2^-$	+++	0	2 (10.5)	2 (4.2)
$cagA^-/vacA^-/oipA^-/iceA2^+$	+++	0	2 (10.5)	2 (4.2)
Total (%)		28 (59.6)	19 (40.4)	47 (100)

[a]The results are cited from our previous paper [24]. *H. pylori: Helicobacter pylori*, PUD: Peptic ulcer disease, VacA: Vacuolating cytotoxin A, OipA: Outer inflammatory protein A gene, CagA: Cytotoxin-associated gene A

CONCLUSIONS

In general, these data support the hypothesis that there is a difference between bacterial strains associated with and without illness. The absolute separation is suggesting that other factors must play a role in the pathogenesis of the disease. Analysis of allelic types virulence genes useful in Iraq and may provide certain combinations of virulence factors are excellent positive or negative marks.

Most *H. pylori* genotypes which associated with peptic ulcer and gastritis were moderate virulent strains, whereas the virulent strain which associated with peptic ulcer belong to, Western *cagA* strains had *vacAs1bm2* genotype, *oipA* and *iceA2* genes which rarely induced gastric cancer in the middle region of Iraq. The *cagA* gene significantly associated with PUD, but it is not related with *iceA*. Both *vacA* and *iceA* related to *oipA* gene.

In Iraq, the *H. pylori*-associated diseases need further molecular studies for bacterial genotypes evaluation to clarifying their role in the clinical outcome and improve molecular diagnoses methods to prevent the bacterial infection risks.

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