

Detection of, *cagA* and *vacA* *Helicobacter pylori* Virulence Genes in Gastric Biopsies of patients with Gastroduodenal disease using Polymerase chain reaction (PCR) technique

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Abstract

The objective of current study was to detect the prevalence of *Helicobacter pylori* by identifying 16SrDNA and to determine the virulence genes (*ureA*, *cagA* and *vacA*) in biopsy specimens from patients suffering gastroduodenal disease using polymerase chain reaction (PCR). Forty samples were obtained by gastroenterologists during endoscopy from gastric antral of suspected individual attending endoscopy unit at Baqubah Teaching Hospital, Diyala, Iraq, during the period from September 2015 to February 2016. According to the endoscopic finding the patients were allocated into four groups of gastroduodenal diseases and control, which include gastritis (GS), duodenal ulcer (DU), gastric ulcer (GU), gastric cancer (GC), their rates were 30% (12), 20% (8), 17.5% (7), 7.5% (3) and 25% (10), respectively. DNA was extracted from the biopsies and subsequently used for PCR detection of *H. pylori* and the virulence genes using specific primers. The results shows that 60% of samples were positive for *H. pylori*, of these positive samples, 91.66%, 66.66%, and 48.83%, were shown to have the virulence genes, *ureA*, *cagA*, and *vacA*, respectively. It is important to mention that *cagA* shown the highest prevalence rate in gastric cancer cases in comparison with *vacA* gene. further studies are required to study the link between *cagA* gene and

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gastroduodenal diseases. In conclusion the result of present study provide important information concerning the prevalence of virulence genes of *H. pylori*.

Keywords: *Helicobacter pylori* , PCR , Virulence genes, *ureA* gene, *cagA* gene, *vacA* gene, Gastrointestinal disease.

التحري عن جينات ضراوة البكتريا الحلزونية *ureA*, *cagA* and *vacA* في خزعات نسيجة من معدة مصابين بأمراض المعدة والاثني عشر باستخدام تقنية تفاعل الكوثرية (PCR)

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الخلاصة

هدفت الدراسة الاستدلال على وجود البكتيريا الحلزونية *Helicobacter pylori* بالكشف عن المورث مشفر الرنا الرايبوسومي 16 أس (16SrDNA) وتحديد امتلاك هذه البكتيريا لمورثات الفوعة: المورث المشفر للوحدة A من أنزيم اليوريز (*ureA* gene) و المورث المقترن بالسمية الخلوية *cagA* ومورث تشفير سم التحوصل الخلوي *vacA* ، في عينات خزعة النسيج من مصابين بأمراض المعدة والاثني عشر باستخدام تفاعل سلسلة أنزيم الكوثرية (PCR). جمعت أربعين خزعة نسيجية بأشراف طبيب الجهاز الهضمي خلال التنظير من غار المعدة من مرضى محالين لوحدة الناظور في مستشفى بعقوبة التعليمي/ ديالى-العراق، خلال الفترة من سبتمبر 2015 إلى فبراير 2016. وفقا لفحص المنظار توزع المرضى الى اربع مجاميع مرضية ومجموعة ضابطة، ضمت التهاب المعدة (GS) وقرحة الاثني عشر (DU)، وقرحة المعدة (GU)، وسرطان المعدة (GC) ، شكلت نسبتها 30% (12)، 20% (8) و 17.5% (7)، و 7.5% (3) و 25% (10) على التوالي. استخلص الحمض النووي الريبوزي منقوص الاوكسجين من خزعات النسيج وكشف عن البكتيريا وعن مورثات الفوعة بتقنية تفاعل سلسلة أنزيم الكوثرية PCR باستخدام بادئات محددة. بين الكشف ان 60% من خزعات النسيج في الأمراض المذكورة أعلاه موجبة للبكتريا الحلزونية، وكانت معدلات تواجد مورثات الفوعة في العينات الموجبة 91.66% و 66.66% و 48.83% للمورثات *ureA* gene و *cagA* gene و *vacA* gene على التوالي. ومن الأهمية بمكان الإشارة الى ان المورث *cagA* gene أعطى أعلى نسبة في حالات سرطان المعدة مقارنة بالمورث *vacA* gene ، وخلص إلى احتمال ارتباط المورث بالمرض. ان هذه الدراسة توفر معلومات هامة بشأن انتشار مورثات فوعة البكتريا الحلزونية.

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كلمات البحث: البكتريا الحلزونية *Helicobacter pylori*، (PCR)، جينات الفوعة *ureA*، *gene*، *cagA gene*، *vacA gene*، أمراض الجهاز الهضمي.

Introduction

Helicobacter pylori is a gram negative and spiral-shaped bacterium which colonizes the human stomach mucoid lining ⁽¹⁾ and linked with increased risk for numerous disease "chronic gastritis(GS), duodenal ulcer (DU), gastric ulcer (GU), dysplasia, mucosal-associated lymphoid tissue (MALT) lymphoma and gastric cancer (GC)"⁽²⁾.

Although World Health Organization (WHO) classified this pathogen as a class I carcinogen ⁽³⁾, a low percentage of the general population establish severe outcome. Which may be due to the specific virulence genes which are responsible for the pathogenicity of the bacterium. Vacuolating cytotoxin which considered as the marker for peptic ulcer disease is encoded by (*vacA*) gene. The *cagA* gene "cytotoxin-associated gene" which is considered as the marker for *cag* pathogenicity island, is associated with peptic ulcer and gastric cancer ^(4,5). The *ureA* gene " encodes for 30 kDa subunit of urease enzyme, that important for bacterial colonization of the human gastric mucosa " is an important *H. pylori* virulence factor ⁽⁶⁾. Recent studies indicate that there are health benefit linked with *H. pylori* colonization to human stomach including protection from obesity, diarrheal diseases, gastroesophageal reflux diseases, allergic airway diseases and Barrett's esophagus, indicating the dynamic and complex link between human and *H. pylori*⁽⁷⁾.

The aims of present study was to determine the prevalence of *H. pylori*, and to detect the virulence genes (*ureA*, *cagA* and *vacA*) using PCR technique.

Material and methods

Study Patients and specimen: Forty individual with dyspeptic symptoms attending endoscopy unit after specialist physician requests. / Baqubah Teaching Hospital in Diyala governorate, Iraq, from the period of September 2015 to February 2016. (23 male and 17 female; mean age, 43.4 ± 1.5 years) were involved depending on the following study criteria:

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- Patients with chronic strong epigastric pain, melena, haematemesis, family history of CA stomach were included.
- Patients with long-term use of (NSAIDs) drugs, anti-*H. pylori* therapy or bismuth containing drugs were excluded.

A detailed history of each patient was obtained concerning, age, gender, address, job, smoking and alcoholism, chronic disease, treatment, admission diagnosis of all study patients. The endoscopic diagnosis grouped them into five categories "gastritis(GS), gastric ulcer (GU), duodenal ulcer (DU), gastric cancer (GC) and control with non-ulcer dyspepsia (NUD), NUD were defined as individual who had no endoscopic lesions (ulcers or malignancies)". During each endoscopic examination, antral gastric biopsy specimens were excised for total genomic DNA extraction. The study protocol was approved by the ethics and research committees of the hospital, and consent forms were obtained from all patients.

Molecular detection of *H. pylori*

Genomic DNA extraction: Biopsy Samples were kept in normal saline and preserved in deepfreeze at -20°C for further analysis. Specimen were thawed, crushed and then genomic DNA was extracted using genomic DNA extraction kit (Geneaid. Chaina). genomic DNA was eluted in 200 μL of $1 \times \text{TE}$ buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]), DNA purity and concentration (ng/ μL) were checked by absorbance at (260 /280 nm) using Nanodrop spectrophotometer (THERMO. USA). The extracted DNA was kept at -20°C until use.

PCR analysis: A specific primer sequences for the gene 16S rRNA (1500bp) was used to confirm the presence of *H. pylori* in the biopsies specimens(Table 1), another sequences of primers (Bioneer. Korea)were used for amplification of the genes *cagA* (1320bp), *ureA* (411bp) and *vacA*(678bp) (Table 1).

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Table1. The primers sequence used in this study

Primer	Sequence (5'.....3')	size	Source
<i>16SrRNA</i>	ATCCTGGCTCAGAGTGAACG	1500bp	(9)
	GCAGGTTACCTACGGTTACC		
<i>ureA</i>	F AGCGTTGGCAGTGCTAAAAG	411bp	(8)
	R TTATAAGCCGCGCCATTAGC		
<i>vacA</i>	F ATGGAAATACAACAAACACAC	678bp	(8)
	R CTGCTTGAATGCGCCAAAC 3		
<i>cagA</i>	F TGATGGCGTGATGTTTGTGA	1320	(10)
	R TCTTGAGGGCGTTGGTGTATT		

The PCR master mix components (AccuPower[®] PCR PreMix Kit) placed in standard AccuPower PCR PreMix Kit that containing all other components which needed for PCR reaction "Taq DNA polymerase, dNTPs, Tris-HCl pH: 9.0, KCl, MgCl₂, stabilizer and tracking dye" as shown in Table2.

Table (2): Mixture of PCR.

PCR Master mix	Volume
DNA template	5µL
Forward primer (10pmol)	1.5ul
Reveres primer (10pmol)	1.5ul
PCR water	12ul
Total volume	20ul

PCR tubes prepared according to the manufacturer instructions, transferred into spin vortex, centrifuge at 3000rpm for 3 minutes, then placed in PCR Thermocycler (MyGene, Bioneer. Korea). PCR amplification conditions for 16SrRNA gene involved: 3 min of pre incubation at 94°C, followed by 35 cycles of 30 s at 94 °C, 30 s at 58 °C, 2 min at 72 °C and 10 min at 72°C for final extension. The *ureA* gen was performed with the following conditions: 5 min of pre incubation at 94°C, followed by 35 cycles of 1min at 93 °C, 30 s at 55 °C, and 1min at 72 °C and 10 min at 72°C for final extension. The *cagA* gen was performed with the following conditions: 5 min of pre incubation at 95°C, followed by 42 cycles of 1min at 95 °C, 1min at 65 °C, and 1min at 72 °C and 5min at 72°C for final extension. The *vacA* gen was performed

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with the following conditions: 5 min of pre incubation at 94°C, followed by 35 cycles of 30 s at 94 °C, 30 s at 56 °C, 30 s at 72 °C and 3min at 72°C for final extension. PCR products were visualized by electrophoresis on 1.5% agarose gels with ethidium bromide.

Statistics Analysis: A t-test was used to compare groups, Data analysis was performed by using SPSS computing program (Statistical Package for Social Sciences) version 16. ⁽¹¹⁾

Results

Study Patients: 40 patients had been enrolled (23 men and 17 women), whose age range was 15-72 years (The mean age was 43.4 ± 1.5 years). The patients distribution among disease groups of gastritis (GS) , duodenal ulcer (DU), gastric ulcer (GU), gastric cancer (GC) and control were 12 (30%), 8 (20%), 7 (17.5%), 3 (7.5%) and 10 (25%) respectively.

Detection and identification the virulence genes of *H. pylori*: The PCR protocol was optimized with using specific primers for 16S rRNA (1500bp) *ureA* (411bp), *cagA* (1320bp), and *vacA* (678bp) (figure 1). Among the 40 Samples examined, were 24 (60%), 22 (55%), 16 (40%) and 11 (27.5%) positive for the genes 16S rRNA, *ureA*, *cagA* and *vacA* respectively. Sixteen (40%) of the tested samples were negative for the said genes that are checked by PCR (Figure 1).

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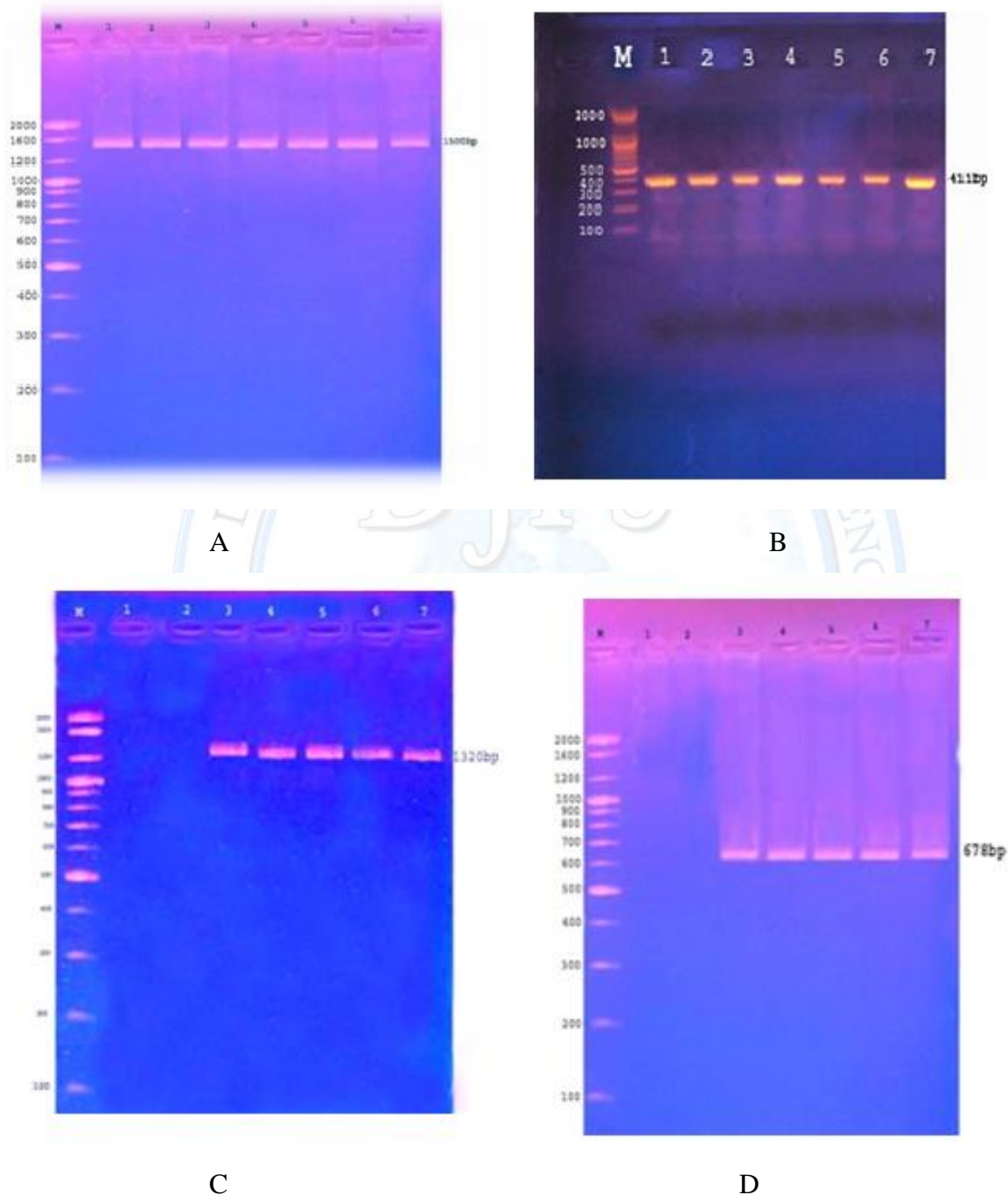


Figure 1: Gel electrophoresis of PCR product A: (16S rRNA 1500bp), B: (*ureA* 411bp), C:(*cagA* 1320bp) and D: (*vacA* 678bp) genes for *H. pylori* . run on a 1.5% w/v agarose gel, 80 volt/cm, for 1 hour, stained with ethidium bromide.

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Virulence genes distribution among groups of diseases

Depending on *H. pylori* 16S rRNA, of 40 biopsies screened only 24 of them were positive for *H. pylori*. According to PCR; 91.66% (22) of the 24 positive biopsies showed that they carry *ureA*, 66.66 % (16) of them carry *cagA*, and 48.83% (11) carry *vacA* (Table3). Two of the 24 positive specimens were found negative in all three virulence genes. this suggests the presence of negative strains of *H. pylori* for virulence genes. The correlation of presence of *cagA* and *vacA* with the evolution of diseases was statistically significant, whilst the differences in the involvement of *ureA* and 16S rRNA genes among diseases group, were not significant

Table (3) distribution of *H. pylori* virulence genes among groups of diseases.

Diseases	16S rRNA(%)	<i>ureA</i> (%)	<i>cagA</i> (%)	<i>VacA</i> (%)
GS	6/12(50)	6/6(100)	4/6(66.66)	2/6(33.33)
DU	7/8(87.5)	6/7(85.7)	3/7(42.85)	4/7(57.14)
GU	6/7(85.7)	6/6(100)	6/6(100)	5/6(83.33)
GC	3/3(100)	3/3(100)	3/3(100)	0/3(100)
Normal	2/10(20)	1/2(50)	0/2(0)	0/2(0)
Total	24/40(60)	22/24(91.66)	16/24(66.66)	11/24(48.83)
<i>P</i> value				

Discussion

Pathological cases distribution: The endoscopy revealed high prevalence of gastritis (GS), duodenal ulcer (DU), gastric ulcer (GU), gastric cancer (GC) among the investigated patients. These results were supported by other study on Iraqi patients⁽¹²⁾ which showed almost similar finding (23%,17%, 20% and 3% of GS, DU, GU and GC, respectively). However, in the cases of GS, GU and DU; results are disagreement with the finding of other workers ⁽¹³⁾ who showed lower prevalence of these diseases. It seems that the different gastroduodenal diseases among patients were varied, which may depend on the selection of patients and/ or the environmental factors.⁽¹⁴⁾

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Distribution of *H. pylori* virulence genes (*ureA*, *cagA* and *vacA*) among gastroduodenal diseases groups: *H. pylori* virulence genes detection by PCR technique has shown great deal of variation ranging from 0-80%.⁽¹⁵⁾ These depend on the target genes used in the analysis. The first targets recruited were urease operon *ureA*.⁽¹⁶⁾ Results of PCR demonstrated a wide range of detection rate among the various investigated genes as well as samples underwent the analysis. It has been showed that (91.66%) of positive *H. pylori* samples were positive *ureA* gene (also show various ratios in gastritis, gastric ulcer, duodenal ulcer and gastric cancer, Table 3). The reason of difference in ratio of *ureA* gene among gastroduodenal disease belong to function of this gene which leads to urea hydrolysis and urease may be released from *H. pylori* to the surrounding area and protects this bacteria from the humeral immune response. It is found in the membrane of *H. pylori* cells and cytoplasm.⁽¹⁷⁾ Among 24 *H. pylori* positives samples, 16(66.66%) carry *cagA* gene. In *cagA* positivity, there were significant differences among different types of gastroduodenal diseases that are investigated, this further substantiates the *cagA* role as a marker for increasing of *H. pylori* virulence (Table 3). The prevalence of *cagA* positive strains of *H. pylori* is varied according to geographical regions. The ratios vary from low (in many Middle Eastern countries, for example, 26.4% in Jordan, 53% in Kuwait, 60.8% in Saudi Arabia⁽²¹⁾), to very high among other Middle Eastern and East Asian countries,(for example, 73.6% in Iran⁽²³⁾ and 92% in Turkey⁽²²⁾, 90% in China⁽¹⁸⁾, 94% in Malaysia⁽¹⁹⁾, 97% in Korea⁽²⁰⁾). the low prevalence of the *cagA* positive strain of *H. pylori* in Iraqi patients could be correlated with the rare incidence of gastric cancer in Iraq. The age-standardized rate (ASR) for gastric cancer incidence in Iraqi men $5/10^5$, which is much lower than that in East Asian countries, e.g. $69.2/10^5$ in Japan and $70.02/10^5$ in South Korea.⁽¹⁴⁾ Despite of the geographical proximity of Iran and Turkey to Iraq, the gastric cancer incidence are varied considerably among these countries (being $8.9-14.1 / 10^5$ and $38-69 / 10^5$, respectively)⁽²³⁾, this is matched with the rate of *H. pylori cagA* positive strains. The functional type IV secretion system, which is encoded by *cagA* island permits the translocation of *cagA* protein into gastric epithelial cells⁽²⁴⁾. that shows high percentage of *cagA* protein, which is associated with peptic ulcer disease, gastric cancer, and

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mucosa-associated lymphoid tissue lymphoma in the stomach⁽²⁵⁾. The presence of the *cagA* pathogenicity island has been associated with more severe *H. pylori* disease⁽²⁶⁾.

The study results of the ratio for the *vacA* gene demonstrated that (48.8%) of the *H. pylori* was positive. Various ratios were associated with pathogenic cases (Table 3). The reason of the difference in *vacA* gene ratio among gastroduodenal disease explained by the action of this gene which acts on formation of acidic vacuoles in the cytoplasm of gastric epithelial cells by creation of pores in epithelial cell membranes of gastric cell, which allows the anions and urea to be released. The *vacA* also induces loosening of tight junctions of gastric epithelial cells, and may allow nutrients to cross the mucosal barrier to *H. pylori*⁽²⁷⁾.

Conclusion

H. pylori bacterium can directly detected from biopsy samples obtained from patient with gastroduodenal diseases using PCR. The study showed a various relationship between gastropathological cases and virulence genes (*ureA*, *cagA* and *vacA*). Only *vacA* was not detected in gastric cancer biopsy and hence, it should be extensively focusing on the mechanism of action of the *ureA*, *cagA* genes in cancer diseases. Therefore, determination of *H. pylori* virulence genes may reveal information about regarding the risk and clinical outcomes in symptomatic patients with gastroduodenal diseases.

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