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Multiplex PCR assay for detection of *Helicobacter pylori* isolated from Iraqi dyspeptic patients

Elham A. Kalaf [1], Nahi Y. Yassen[2], Zahra M. AL-Khafaji[1], Ghaith L. A'Rif [3], Bashar A. Abass[4]

1 *Genetic Engineering and Biotechnology Institute for Post Graduate Studies, Baghdad University.*
2 *Iraqi Center of Cancer and Medical Genetics Researches, Almustanseria University.*
3 *Al-Nahrain Forensic DNA Training Center (NFDTC).*
4 *Al-Kadhimiya Teaching Hospital.*

Abstract:

Helicobacter pylori (H. pylori) is one of the most common infections worldwide and is associated with gastric disorders. H. pylori is genetically unstable and this reflected on its virulence factors and type of diseases. Cytotoxin associated gene A (CagA) product is a major virulence factor is thought to be associated with gastric disease. It is injected into epithelial cells, undergoes phosphorylation by host cell kinases, and perturbs host signaling pathways. In the present study, we used multiplex PCR assay for rapid detection of H. pylori infection and for the determination of CagA genotypes directly from gastric biopsy specimens. Gastric biopsies were collected from 210 patients with dyspeptic symptoms. Genotypic analysis of H. pylori genes was found 91.17% for 16S rRNA, 87.25% for flagellin A, 89.21% for glmM (ureC) and cagA gene was detected in (39.21%) 40 biopsy specimens. Our study indicated that ureC (glmM) gene PCR is the most specific for the detection of H. pylori in gastric biopsy specimens compared with16S rRNA gene. There were significant differences ($P \leq 0.01$) in cagA positive rate, among different diseases.

Keywords: *Gastric cancer, Multiplex PCR, CagA, glmM (ureC), 16S rRNA*

Introduction:

Helicobacter pylori is a rather recently discovered bacterium that was first isolated from a gastritis patient in 1982 (1). H. pylori is a Gram-negative, spiral shaped, and fastidious microaerophilic bacterium that chronically colonizes the gastric epithelium of more than half of all people worldwide (2). It is a human pathogen responsible for chronic active gastritis, and infection with this organism is an important risk factor for peptic ulcer, gastric cancer, and gastric mucosa-associated lymphoid tissue (MALT) lymphoma and adenocarcinoma (3). Due to this correla¬tion, H. pylori is the only bacterium currently classified as a class I carcinogen by the World Health Organization (4). Indeed, gastric cancer is still the second most common cause of worldwide cancer mortality, and this high rate could be reflective of the high incidence of H. pylori infection (5).

Corresponding Address:
Elham A. Kalaf
Genetic Engineering and Biotechnology Institute for Post Graduate Studies, Baghdad University.
Email: elhamkalaf@yahoo.com

H.pylori clinical isolates are classified into two types according to their degree of pathogenicity. Type I is associated with severe disease pathology contains vacA and Cag Pathogenicity Island (cag PAI), Type II lacks cagPAI, have non-toxic form of vacA, and regarded, as a virulent (2). cagA gene is one of the most studied virulence factors of H.pylori, the presence of cagA is considered a marker of the presence of cag PAI (2,5). cagA is the last gene on the cag PAI, and encodes for the 120–145 kDa immunodominant cagA protein (6). Infection with cag PAI-positive H. pylori is statistically associated with duodenal ulceration, gastric mucosal atrophy, and gastric cancer (7). H.pylori is a highly heterogeneous bacterium with a large genomic diversity. In addition, humans may sometimes harbor multiple strains. H. pylori can change genotypically and phenotypically during colonization in a single host (8). Strain-specific diversity has been proposed to be involved in the organism's ability to cause different diseases; there are also indications of significant geographical differences among strains (9).

Multiplex PCR assay methods have been developed to detect the organism directly in clinical specimens. The targets of these PCR methods include the 16S rRNA, flagellin

A, glmM (ureC) and CagA genes. The 16S rRNA is one of the specific targets to confirm H. pylori specific DNA which may be considered as a direct evidence of the presence of the pathogen (10). The glmM (ureC) gene encodes for a phosphoglucosamine mutase; this gene is unrelated to urease production, so it was renamed glmM. (11) This gene is considered a “housekeeping gene” and it participates directly in cell wall synthesis. There is only one copy of the ureC gene per bacterium and thus ureC gene appears to be specific for H. pylori and used for primary identification of the organism (11, 12). Flagellin encoded by flagellin A gene is required for (i) bacterial colonization, (ii) bacterial survival in the stomach mucus, and (iii) display of active motility in viscous environments inhibitory to the motility of other bacteria (13). The aims of this study were to determine the prevalence of Helicobacter pylori from dyspeptic patients in Iraq and to determine CagA gene among H. pylori isolates.

Materials and Methods:

Patient-derived Samples

A total of 210 Iraqi patients (79 men and 113 women; mean age, 44.4 ± 1.6 years) with dyspeptic symptoms were included. They underwent endoscopy in Al-Kadhimiya teaching hospital and Al-yarmook hospital in Baghdad, Iraq between June 2010 and August 2011. The endoscopic diagnosis grouped them into three categories: peptic ulcer (gastric ulcer (GU) and duodenal ulcer (DU)), gastric cancer (GC) and control with non-ulcer dyspepsia (NUD). NUD patients were defined as patients who had no endoscopic lesions of ulcers and/or malignancies. After endoscopic examination, the gastric biopsy specimens from the antrum were examined for the presence of H. pylori by rapid urease test and PCR. The study protocol was approved by the ethics and research committees of the hospital, and all patients gave informed consent to the study.

Processing of the Samples for PCR Assay

One antral biopsy specimens from each person were kept in normal saline (0.9% sodium chloride) and preserved immediately at −80°C for molecular analysis. Each frozen biopsy specimen was thawed, crushed and then genomic DNA was extracted directly from gastric biopsy samples using the QIAamp tissue DNA extraction kit (QIAGEN, Hilden, Germany) according to the manufacturer’s instructions. The extracted DNA then quantified through measurement of its OD260 by ND-2000 spectrophotometer (Thermo Scientific Inc., USA). The extracted DNA was eluted in 200 µL of 1 × TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]) and stored at -20°C until use.

PCR amplification analysis

In order to confirm the presence of H. pylori in the biopsies under investigation, a specific primer sequences for the genes cagA (349bp), ureC (315bp), flagellin A (152bp) and 16S rRNA (110bp) (Maxim Biotech.Inc. USA). A Master Mix components of MPCR was adopted (Table 1).

Table 1: The Master mix components of MPCR.

Component	Concentration	Amount (µl)
Deionized water	__	14.5
MPCR Buffer Mixture	2X	25.0
MPCR Primers	10X	5.0
Taq DNA Polymerase	5U/µl	0.5
Specimen DNA or Control DNA from kit	(200ng/ µl) 10X	5.0
Total volume	__	50

Polymerase chain reaction was performed in a thermocycler (MJ MINI, Bio-Rad. France) under the following conditions adopted in (Table 2).

Table 2: The MPCR cycles for detecting the presence of H.pylori genes cagA, ureC, flagellin A and 16S rRNA in patient biopsies.

No.	Step	Temperature (°C)	Time	No. of Cycles
1	Initial denaturation	96	.min 1	2
	Initial annealing and extension	60	.min 4	
2	Denaturation	94	1min	35
	annealing and extension	62	2min	
	Final extension	70	.min 10	1

The MPCR products and the ladder marker (5µl) were resolved by electrophoresis. 2µl of loading buffer plus 10 µl of the product were loaded on 2% agarose gel containing 2µl ethidium bromide (5 mg/ml) and run at 6volt /cm for 2 hour. Finally, bands were visualized on UV transiluminator and then photographed using photo documentation system (Scie-Pluse, American science and surplus, USA). All experiments were performed in triplicate.

Statistical analysis: The differences in presence genes of H. pylori and disease groups (gastric ulcer, duodenal ulcer, gastric cancer, and gastritis patients) were analyzed using a $\chi 2$ test. Statistical significance was tested at $P < 0.01$.

Results:

Characteristics of patients

The mean age of the 210 patients (113 women and 97 men) enrolled in the present study was 44.4 ± 1.6 years with a range of 15-72 years. The median age was 43.10 years.

The patients distributed among of groups of gastritis , gastric ulcer (GU), duodenal ulcer (DU), gastric cancer (GC) and control in 87(41.42%), 39(18.57%), 52 (24.76%), 12 (5.71%) and 20 (9.52%) respectively.

Prevalence of H pylori infection in Iraqi populations:

1-Detection of H pylori infection using rapid urease test (RUT):

This test was conducted immediately after obtaining the biopsy from the patient. Urea agar became pink in the presence of H. pylori urease .The time taken for the positive reaction was one minute to 24 hour. Rapid urease test revealed that 102 out of 210 patients (48.57%) were infected by H. pylori, while 108 patients (51.43%) did not exhibit the presence of H. pylori and considered negative. The decrease of H. pylori positive samples was significant ($P \leq 0.01$) with respect to H. pylori negative samples.

2- Detection of H. pylori using Multiplex PCR (MPCR):

Amplification and melting conditions were optimized for the MPCR assay, using specific primers sequences of cagA (349bp), ureC (315bp), flagellin A (152bp) and 16S rRNA (110bp) (Figure 1). Out of the 210 biopsies screened, 97 (46.01%) were positive for three genes 16S rRNA, flagellin A and ureC, from this 97 biopsies only 40 (19.04%) were positive for cagA. One hundred and thirteen (53.80%) of the biopsies screened were negative for all four genes screened in MPCR methods.

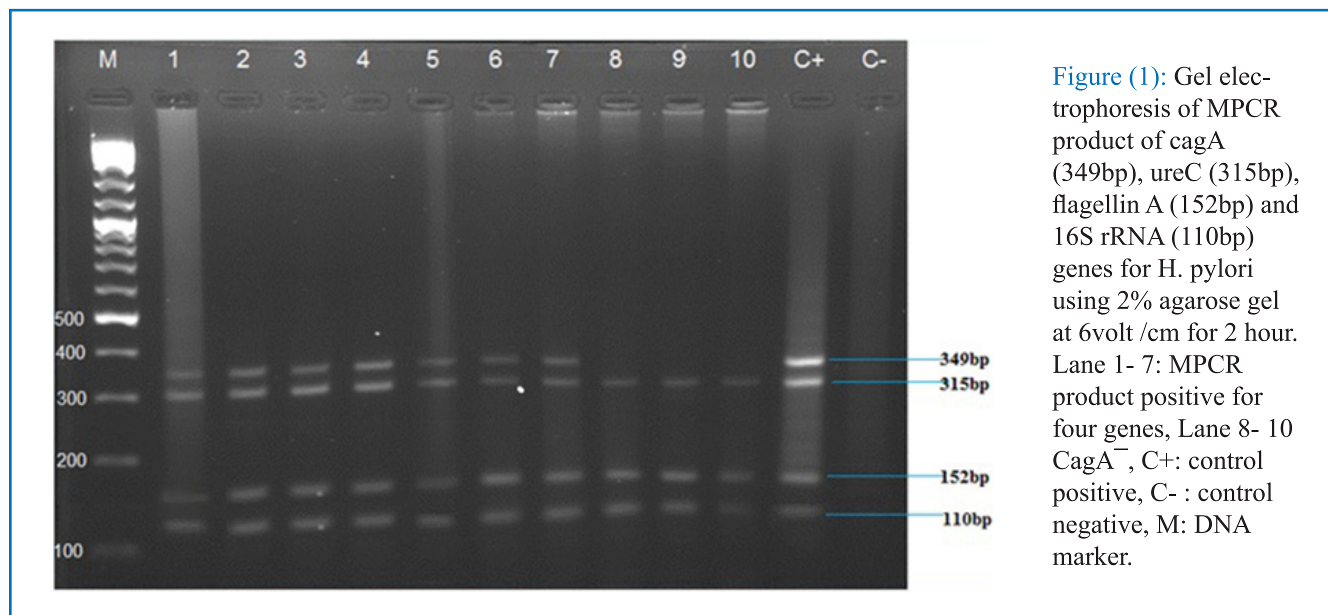

Figure (1): Gel electrophoresis of MPCR product of cagA (349bp), ureC (315bp), flagellin A (152bp) and 16S rRNA (110bp) genes for H. pylori using 2% agarose gel at 6volt /cm for 2 hour. Lane 1- 7: MPCR product positive for four genes, Lane 8- 10 $CagA^{-}$, C+: control positive, C- : control negative, M: DNA marker.

- Distribution of genes among disease groups

In 210 biopsies screened, H. pylori was observed at a highest rate in patients with DU and patients with Gastritis, then patients with GU according to presence of the three genes 16S rRNA, flagellin A and ureC. On the other hand the lowest rate was observed in the patients with GC.

- Distribution of genes among disease groups in positive H.pylori specimens

The genes distribution according to MPCR, showed that in 102 biopsy of the H.pylori positive, found 100 (99.09%) carried 16S rRNA, 98 (96.07%) flagellin A, 97 (95.09%) ureC and 40 (39.22%) carried cagA (Table 3). Two of the 102 positive specimens (according to positive result in RUT) were found negative in all four genes in MPCR. We suggested that the positive results of RUT obtained may belong to urease producing bacteria other than H. pylori. In addition H. pylori colonization in gastric mucosa is often patchy rather than uniform (14), the separate biopsies used for MPCR and RUT may harbor two different strains.

The relation between the presence of both ureC and cagA and the development of diseases was statistically significant ($P \leq 0.01$) while there were no significant differences in presence of 16S rRNA, flagellin A among different diseases.

Table 3: Distribution of H.pylori genes among disease groups of dyspeptic patients.

Disease	16S *rRNA* (%)	*Flagellin A* (%)	*ureC* (%)	*cagA* (%)
Gastritis	34/30 (88.23)	(94.11) 32/34	(94.11) 32/34	6/34 (17.6)
GU	22/23(95.65)	22/23 (91.30)	(95.65)22/23	23/10(43.4)
DU	45/43 (95.55)	(88.84)40/45	(88.84)40/45	45/21(46.7)
GC	(100)3/3	(100)3/3	(100)3/3	3/3(100)
Normal	2/2(100)	2/2(100)	2/2(100)	0/2(0)
Total	(98.09) 100	(96.07) 98	(95.09) 97	(39.22) 40
X^2 –test	1.01	1.02	83.8	75.67
***P* Value**	N.S	N.S	P≤0.01	P≤0.01

Discussion:

In recent years, many investigators used molecular techniques to reveal that H. pylori possess a remarkable degree of genetic diversity. This diversity is closely related with its epidemiological and pathological characteristics and dynamic of transmission (8). The MPCR analysis has been widely accepted for typing and differentiation of H. pylori strains from clinical specimens (12, 15). This method has been used to identify conserved H. pylori genes, especially 16S rRNA, flagellin A and ureC . H. pylori usually does not cause illness, but colonization with strains bearing the cag pathogenicity island (cag PAI) (2) is associated with an increased risk of gastric adenocarcinoma and peptic ulcer disease (3, 5).

In the present study, both of the used genes 16S rRNA and ureC are housekeeping genes (16). The distribution of ureC gene (95.09%) was lower than that of the 16S rRNA gene (98.09%). This result suggests that ureC (glmM) gene is more convenient for the detection of H. pylori in gastric biopsy specimens with PCR. This finding is consistent with the previous report indicated that 16S rRNA gene PCR nonspecifically amplifies human DNA (16). Although similar results were also noted by de Reuse et al.(17) when observed unexpected positive results of H. pylori 16S rRNA gene PCR applied to human tissue specimens (liver biopsy specimens and colon biopsy specimens) indicates nonspecific amplification of human genomic DNA by H. pylori 16S rRNA gene primers. However, the 16S rRNA gene PCR has been the most widely used method for the detection of H. pylori in clinical specimens (12). The results of Lu et al. (15) give support to the result of the present study; they mentioned that ureC (glmM) gene PCR is the most specific for the detection of H. pylori in gastric biopsy specimens compared with 16S rRNA gene. The ureC (glmM) gene is essential for the development of the cell wall in bacteria as well as for the growth of the microorganism, and this gene has been extensively used for confirming the presence of H. pylori (15,18).

The prevalence of H. pylori infection varies between countries; generally, the prevalence is about 30% in developed and up to 80% in developing countries (19). Our investigation arrayed H.pylori presence in (46.01%) of patients come in concordance with some previous works that study the prevalence of H. pylori in Iraqi patients (20,21) .While prevalence of H. pylori increased markedly in countries geographical proximity to Iraq in Turkey and Iran 88% and 74.2% respectively (22,23).

Among 102 H. pylori positives, 40 (39.22%) were positive for cagA gene. There were significant differences (P ≤ 0.01) in cagA positive rate, among different type of gastric diseases under investigation, this further substantiate the role of cagA as a marker for increased virulence of H. pylori (Table 3).

The prevalence of cagA positive H.pylori varies from one geographic region to another. The rate differs from very high in East Asian countries to low from many Middle Eastern countries. For example, 97% in Korea (14) , 94% in Malaysia (24), 90% in China (25). And among H. pylori strains isolated in the Middle East , 60.8% in Saudi Arabia, 26.4% in Jordan , 53% in Kuwait (26), 73.6% in Iran (22), 92% in Turkey (21) . Low prevalence of strains with cagA positive in Iraqi patients might be reasons for low incidence of gastric cancer in Iraq. The incidence of gastric cancer in Iraq is rare (age-standardized rate [ASR] for men 5/105) which is much lower than that in East Asian countries (e.g. Japan 69.2/105 and South Korea 70.02/105) (14). Despite the countries geographical proximity to Iraq , Turkey and Iran where the incidence of gastric cancer differs hugely among these countries, being 8.9–14.1/105 and 38–69/105, respectively (22, 23).

Assays with MPCR of genomic DNA extracted directly from biopsy specimens appear to be highly sensitive, allows detecting even few cells per reaction. The method also provides a preliminary H. pylori genotyping directly from biopsy specimens, and could be suitable for large scale diagnosis.

The medical and scientific community anticipates that rapid molecular methods to determine antibiotic susceptibility, bacterial density, and virulence characteristics of H. pylori, as well as patient genotype information, will soon widely accepted (17). These molecular tests rapidly allow treatment to be designed for the individuals, and will be more effective and result in fewer side effects, reduce overall costs, and lead to a decrease treatment failures and development of fewer antibiotic-resistant H. pylori strains. The use of multiple diagnostic methods was recommended to accurately diagnose H.pylori infection (12,17). In summary, we believe the multiplex PCR could be of great value in clinical microbiology and H. pylori population studies, especially for rapid screening of many samples to detect H. pylori infection and to determine cagA status.

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استعمال تقنية تفاعل الكوثرة المتعددة لتشخيص الاصابة ببكتريا الملوية البوابية *Helicobacter pylori* المعزولة من مرضى عراقيين

الهام عبد الهادي خلف[1]، ناهي يوسف ياسين[2]، زهرة محمود الخفاجي[1]، غيث لطفي عارف[3]، بشار عباس[4]

1 معهد الهندسة الوراثية والتقنيات الحيوية للدراسات العليا / جامعة بغداد
2 المركز العراقي لبحوث السرطان والوراثة الطبية /الجامعة المستنصرية
3 مركز النهرين للتدريب على الحامض النووي العدلي
4 مستشفى الكاظمية التعليمي / بغداد

الخلاصة:

تُعد الاصابة ببكتريا الملوية البوابية Helicobacter pylori (H. pylori) الأكثر شيوعا في جميع أنحاء العالم وترتبط الاصابة بهذه البكتريا مع اضطرابات الجهاز الهضمي. تتصف البكتريا بعدم ثبوتها الوراثي والذي ينعكس على عوامل ضراوتها وبالتالي تنوع الامراض التي تسببها. يعد سم CagA من عوامل الضراوة الرئيسة المرتبط بأمراض المعدة ، إذ يؤدي حقنه في حدوث تغيرات في الخلايا الطلائية ويفسفر السم بواسطة عائلة الكاينيزات الخلوية للمضيف مؤثرا في شبكات نقل الإشارات للخلايا. ركزت الدراسة الحالية على استعمال تقنية تفاعل الكوثرة المتعددة (MPCR) Multiplex PCR لتشخيص الاصابة بالملوية البوابية وتحديد النمط الجيني لجين cagA في الخزع النسيجية للمعدة . جُمعت 210 خزعة نسيجية من مرضى عراقيين ظهرت عليهم اعراض اضطرابات الجهاز الهضمي . تضمن التشخيص الجيني تحديد وجود اربع جينات (glmM ureC ، flagellin A ، 16S rRNA) و cagA تراوحت احجامها بين 110 زوج قاعدي و 152 زوج قاعدي و 315 زوج قاعدي و349 زوج قاعدي على التوالي . اظهر الفحص عن جين (glmM ureC) حساسية ودقة اكثر من جين 16S rRNA حسب الطريقة المستخدمة . اظهرت نتائج التحليل الاحصائي على وجود علاقة معنوية ($P \le 0.01$) بين معدل وجود جين cagA واختلاف أمراض الجهاز الهضمي.