Molecular Detection of *Helicobacter pylori* Infection in Gastric Biopsy Specimens by PCR

Haider Ali AlNaji **Rabab Omran**
Department of Biology, College of Science- University of Babylon
Haligeoda@yahoo.com sci.rabab.omran@uobabylon.edu.iq

Ali AlSherify
College of Medicine- University of Babylon

Abstract

Infection with *Helicobacter pylori* is associated with the development of different gastric disorders. Clinical outcome of *H. pylori* infection related to virulence factors that encoded by genes of this bacteria that can be used in a molecular detection such as the housekeeping genes; *ureA* and *ure*C. 16S rRNA is also used in bacterial diagnosis.

Seventy-five patients with dyspeptic symptoms sent to esophago- gastroduodenal scope (OGD) unit at Merjan Hospital in Babylon Province. They were diagnosed by specialist physicians and selected in the current study and were classified 43 patients had gastritis, 23 patients had peptic ulcer disease(PUD), 3 had growth like mass non cancer and 6 were normal as negative control.

The results of multiplex PCR and monoplex PCR revealed that a total of 49 (65.3%) cases were found positive for *H. pylori* by 16S rRNA and *ureA*, whereas *ure*C primer is less sensitive for bacterial detection.

Key words: *Helicobacter pylori*, Molecular Detection, PCR, 16S rRNA, *ureA*, *ure*C

الخلاصة

ترتبط اخماج بكتريا *Helicobacter pylori* بتطور حالات التهابات المعدة والقرحة التي لها علاقة بعوامل الضراوة للبكتريا وهذه العوامل تشفر لها جينات البكتريا التي يمكن استعمالها في التحري عن وجود البكتريا فضلا عن 16S rRNA.

تم جمع 75 عينة من المرضى اللذين لهم اعراض سوء الهضم والمراجعين لودة تنظير الجهاز الهضمي في مستشفى مرجان في محافظة بابل. وتم فحص المرضى واخذ العينات منهم من قبل أطباء أخصائيين. شملت الحالات 43 حالة التهاب المعدة و23 حالة لديهم القرحة المعدية و ثلاث حالات لديهم نمو ورمي و6 حالات عادية استعملت كسيطرة سالبة .

اظهرت النتائج لفحص multiplex PCR و PCR باستعمال بادئ منفرد بان 65,3 % من الحالات موجبة لجينين 16S rRNA & *ureA* في حين اظهر البادئ *ure*C حساسية اقل في التشخيص.

كلمات افتتاحية: الملوية البوابية, التشخيص الجزيئي, بلمرة المتعدد

Introduction

Helicobacter pylori, since the natural ecologic niche of this bacterium is human and animal gastric mucosa and colonizes the gastric mucosa of more than 50% of the human population, there is a large attention from investigators from many disciplines to study these bacteria; due to severe *H. pylori*-associated diseases that developed in acquisition of *H. pylori*, like corpus gastritis, gastric atrophy, gastric ulcer, and increased risk of gastric cancer or gastric mucosa-associated lymphoid tissue lymphoma(MALT), in addition another subset of disease like duodenal ulcer. Moreover *H. pylori*-infected subjects largely developed asymptomatic chronic active gastritis, which then involved increased risk of gastric carcinoma (Stein *et al*., 2013).

Helicobacter pylori previously named *Campylobacter pyloridis*, is spiral-shaped Gram-negative bacteria, 2 to 4 μm long with a diameter of 0.5 to 1 μm. Microaerophilic has 2-6 lophotrichous (unipolar bundle) flagella *H. pylori* genomes which is approximately 1.7 Mbp, with a G+C content of 35 to 40%. With approximately 1600 genes (Oleastro and Ménard, 2013; Zanotti and Cendron, 2014).

There are two methods for detecting the presence of *H. pylori* including invasive and noninvasive each with its own advantages, disadvantages, and limitations. Polymerase chain reaction technique is more powerful method for diagnosis, that allow to detect *H. pylori* in small samples even with low quantity, as well as analyses of diversity and virulence of these bacteria (Garza-González *et al*., 2014). 16S *rRNA*, *ureA* and *ureC* are housekeeping genes and used extensively in detecting the presence of bacteria.

The aim of this study is the diagnosis of *H. pylori* in biopsy specimens using PCR technique.

Materials and Methods

75 patients undergoing gastroduodenal endoscopy from 1 February to 30 Jun 2015 at Endoscopic examination unit in Merjan Hospital were included in the current study.

population consisted of 34 men and women 41 of ages range from 16 to 74 years (mean age 38.5).

The biopsy specimens were collected from patients; 23 of them had peptic ulcer disease(PUD), 3 nodular growths nonmalignant, 6 had normal as control and all other subjects (43) had gastritis, that symptoms either abdominal pain or dyspepsia and had no evidence or history of gastric or duodenal ulcers. The biopsies collected from patients were stored in normal saline and transported on ice to the laboratory for processing or stored at -20 C until analysis.

Extraction of DNA from Biopsy Specimens:

Each frozen biopsy specimen was thawed, crushed; genomic DNA was then extracted directly using FAVORGEN tissue genomic DNA extraction mini kit (USA). DNA quantity and integrity was determined using a spectrophotometer (Nanodrop). The concentration of DNA was measured at ng/ml and the DNA purity estimated by reading absorbance at 260/280nm.

Detection of *H. pylori* Using Multiplex PCR (MPCR):

For confirming the presence of *H. pylori* DNA in biopsies Two sets of primers were used specifying genes *ureC*(294bp), 16S rRNA (110bp) amplification and melting conditions were optimized for the MPCR assay. Multiplex PCR technique in addition to monoplex PCR technique were used to detect *ureC* and 16S *rRNA*

MPCR Working Solution:

Table (1): The Master Mix components of MPCR

Component	Amount (µl)	Concentration
Master Mix	12.5	1X
DNA	5	200ng/µl
16S rRNA primers	2	10pmol
ureC primers	2	10pomol
DNas free water	Up to 25µl	-
Total volume	25µl	-

Primers

The sources of all primers used in this study was Bioneer (south Korea). The name, sequence and product size are given in table (2).

Table (2): The name, sequence and product size of primers used in this study

Gene	Name of primer	Sequence of primer 5'-3'	Product Size (bp)	Reference
16S rRNA	Hp1 Hp2	CTG GAG AGA CTA AGC CCT ATT ACT GAC GCT GAT TGT GC	110	Ho *et al*,1991
ureA	HPU1 HPU2	GCCAATGGTAAATTAGTT CTCCTTAATTGTTTTTAC	411	Santos *et al*., 2003
ureC	GlmM-F GlmM-R	AAGCTTTTAGGGGTGTTAGGGGTTT AAGCTTACTTTCTAACACTAACGC	294	Lu *et al*., 1999

MPCR Protocol:
PCR was performed in a thermocycler under the following conditions adopted in table (3).

Table (3): The MPCR protocol for *16S rRNA*, *ureC*, genes detection.

No.	Step	Temperature °C	Time	cycle
1.	Initial denaturation	95	10 min.	1
2.	Denaturation	95	30 sec.	35
3.	Annealing	55	1 min.	
4.	Extension	72	1 min.	
5.	Final extension	72	5 min.	1
6.	Hold	4	5 min.	1

Agarose gel electrophoresis:

Each reaction product (5 µl) was added to 2 µl of gel loading buffer [0.25% (w/v) xylene cyanol, 30% (v/v) glycerol, 100 mM EDTA, pH 8.0], electrophoresed on a1. 5% agarose gel containing ethidium bromide (0.5 µg/ml), visualized under ultraviolet (UV) illumination, and photographed. A 100-bp DNA ladder (Bioneer - south Korea) was used as a size marker (Sambrook *et al*., 1989)

Statistical 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 significant *P* value of <0.05.

Results and discussion

Characteristics of Patients:

A total of 75 patients with age mean (38.5) year; 34 men and women 41 with age range from 16 to 74 years; participated in current study. The results of gastrointestinal endoscope were 43(57.3%) had gastritis, 23 (30.7%) had peptic ulcer disease (PUD) either gastric ulcer or duodenal ulcer, 3 (4%) had Nodular growth non carcinoma and

6 (8%) had normal as control (Fig.1). The percentage of *H. pylori* to incidence of gastrointestinal disease has been tested, and it shows significant association between presence *H. pylori* and gastritis and PUD ($p \leq 0.0001$) as shown in Fig. (1).

The frequency distribution of males with gastritis, PUD (GU or DU) and nodular growth non cancer was 18/34 (52.9%), 9/34 (26.5%) and 2/34 (5.9%) Respectively, while among females it was 25/41 (61.0%), 14/41 (34.1 %) and 1/41 (2.4%) respectively, Fig. (2).

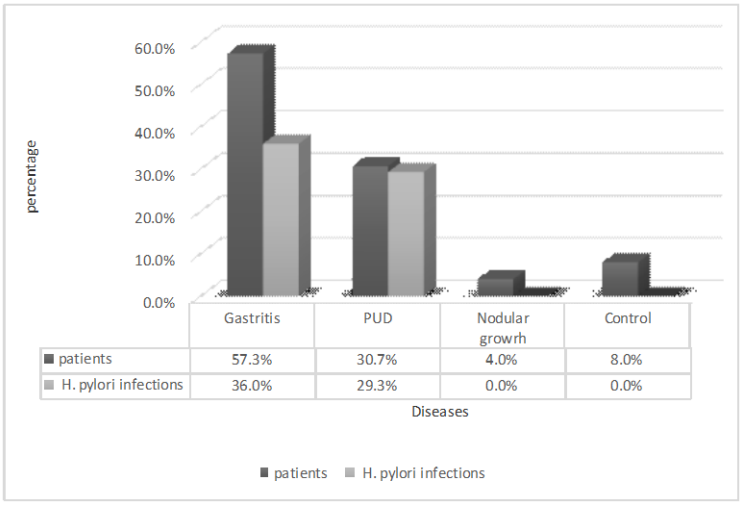

Figure 1: Comparison between diseases and percent of patients that had disease with *H. pylori* infections.

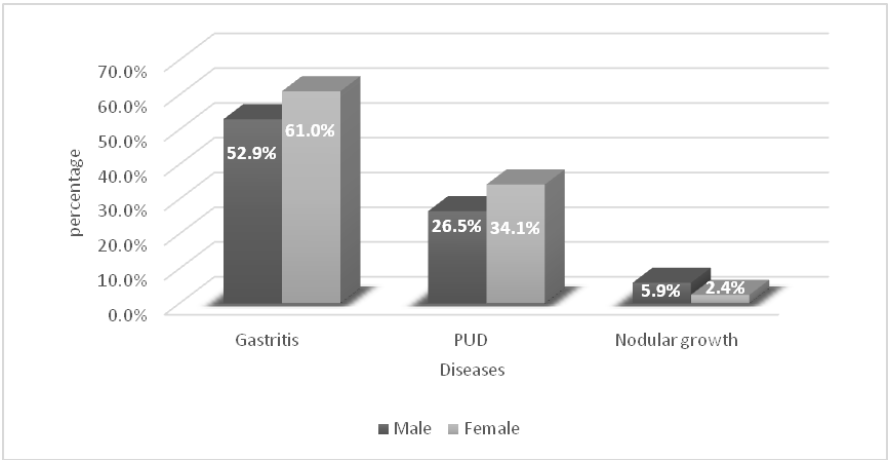

Figure 2: Distribution of patients according to gender with various diseases.

H. pylori infection was more frequent among < 40 years' group rather than > 40 years' group and there is no significant relationship *H. pylori* infection and age groups.

However the greatest percentage of *H. pylori* infections were females (78.05%) and we also found association between presence of *H. pylori* infections and gender ($p = 0.011$), and females are more likely to be infected with this bacterium than males (Table 4). Distribution of diseases and colonization of *H. pylori* among gender may depend on hormonal genetic factors as mentioned by Zheng, (2011), as well as the patients that sent to endoscopy and enrolled in current study.

Table 4: Percentage *H. pylori* infections among patients according to gender and age.

Characteristic		No. of patients	*H. pylori* infections (%)	*P* value
Age (Years)	> 40 < 40 Mean 38.5 SD =17.3	33 42	19(57.6) 30(71.4)	0.157
Gender	Male Female	34 41	17(50) 32(78.05)	0.011

Molecular Detection of *H. pylori:*

Molecular identification of *H. pylori* was applied to all biopsies by PCR using specific primers for primary identify biopsies that contain bacterium. The rate of positive *H. pylori* in the biopsies tested was 65.3% (49/75), depending on all primers used in the study, different studies varied in their results about prevalence of *H. pylori* infection, in middle East, depending on PCR and histopathology, the prevalence was 77.5% and 79% in Jordan and Bahrain, respectively (Fakhro *et al*., 1999; Nimri *et al*., 2006). Other countries had shown low percentage especially in developed countries (Atherton, 2006).

Detection presence of *H. pylori* based on *ureC* and 16S *rRNA* genes:
Multiplex PCR (MPCR):

PCR technique used in detection of DNA from several fastidious microorganisms such as *H. pylori* has been highly recommended (Clayton *et al.*, 1992). In multiplex PCR two sets of primers were used, GlmM and Hp for amplification and 16S *rRNA* respectively; they had the same annealing temperature (55°C) but produced PCR products of different sizes. The results of .*H pylori* genotyping by multiplex PCR were confirmed by single PCR assays of each target sequence, for more emphasis the results.

16S *rRNA* and *glmM*, are housekeeping genes and they are useful to identify *H. pylori* directly in gastric biopsies with high sensitivity (Ho *et al*, 1991), however; the result of PCR (Fig. 3); *glmM* is 19/49 (38.8%) was lower than that of the 16S *rRNA* gene 47/49(95.9%). Our result confirms studies in Egypt where 36% of cases with sensitivity and specificity of 65.9% and 44.4%, respectively. Helaly *et al.* (2009). And *Lage et al.* (1995) reported Similar results (38.5%). Moreover, Lim *et al*. (2003) and *Brooks et al.,* (2004) detected *glmM* by PCR in 48.8% and 44% respectively of the studied cases.

This low percent may be due to sequence polymorphism in the *glmM* gene or/in variation to the diversity of strains within the patients (Mishra *et al.*, 2003; Helaly *et al.*, 2009). As it is reported in previous studies that there is evidence that polymorphisms also exist within the *ureC* gene (Courcoux *et al*., 1990), also geographic regions affects housekeeping genes and point mutations, Intragenic and recombination are another potential factors (Raymond *et al*., 2004), other authors mentioned *ureC* gene conserved and applied PCR for a *glmM* gene segment was almost better than other methods for the detection of *H. pylori* infection, since it has a high degree of sensitivity and specificity (Hoand and Windsor, 2000; Brooks *et al*., 2004; Kalaf *et al.*, 2013). The *ureC* gene encodes phosphoglucosamine mutase, so it is renamed to *glmM* by De Reuse *et al.* in 1997, which is unrelated to urease production.

monoplex PCR:

A: 16S *rRNA* gene:

16S *rRNA* is useful for primary detection use Hp1, Hp2 primers with sensitivity up to 100%, this primer is widely used and has been proven to have a high sensitivity (Ho *et al*, 1991). with product 110pb results of 47 positives out of 49 specimens. With percentage 95.9 % (Fig. 4)

B: *ureC* gene:

Monoplex PCR for *ureC* gene was carried for detection (294bp) PCR product (Fig.5). The results were 19 samples carry *ureC* gene out of 49 cases; (percent 38.8%)

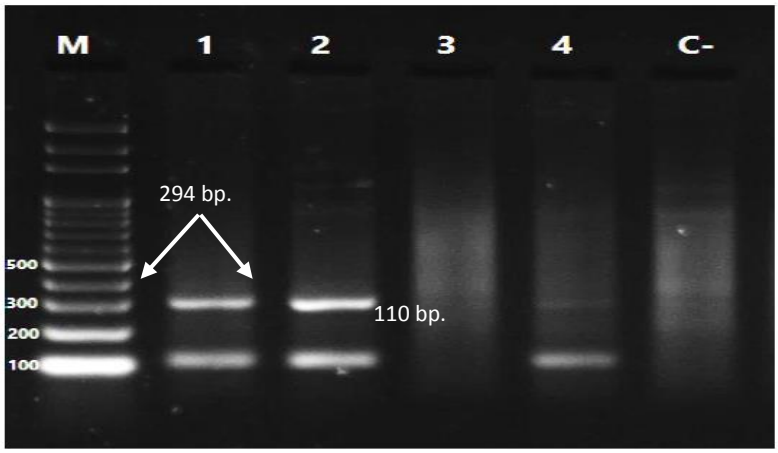

Figure 3: Gel electrophoresis of MPCR product of *ureC (*294bp)*,16S rRNA (*110bp) genes. 1.5% agarose gel at 7volt /cm for 1 hour. Lane 1,2: PCR product positive for 16S *rRNA* and ureC, Lane 4: 16S *rRNA* positive only, Lane 3: negative for all genes, C-: negative control M: 100-bp DNA marker.

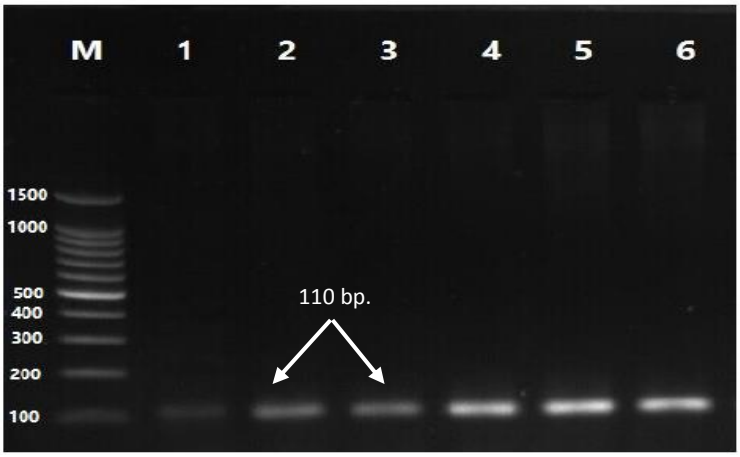

Figure 4: Gel electrophoresis of PCR product of 16S *rRNA* gene (110bp). 1.5% agarose gel at 7volt /cm for 1 hour. Lane 1- 6: PCR product positive for 16S rRNA gene, M: 100-bp DNA marker.

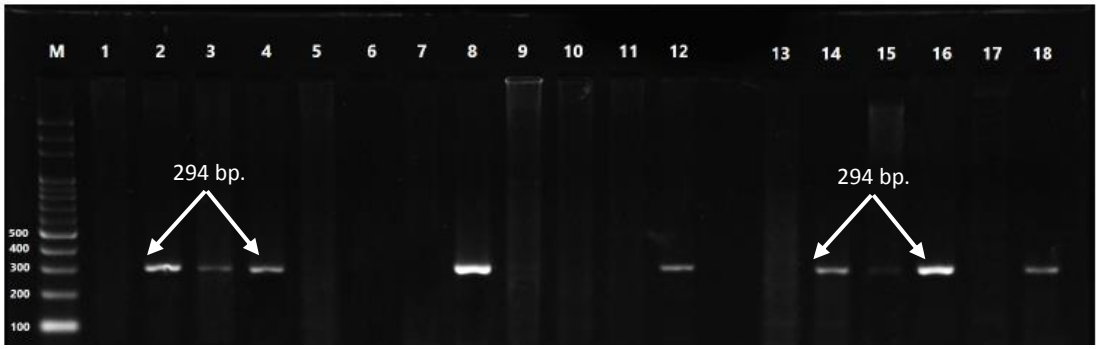

Figure 5: Gel electrophoresis of PCR product of *ureC* gene (294 bp). 1.5% agarose gel at 7volt /cm for 1 hour. Lane 2,3,4,8,12,14,15,16,18: positive for *ureC* gene, others Lane were negative, M: 100-bp DNA marker.

Detection presence of *H. pylori* based on *ureA* gene

PCR with primers HPU1 and HPU2 enabled the detection of *H. pylori* directly from biopsy samples. The results of PCR amplification of *ureA* are shown in (Fig. 6) amplicon size is 411bp, where 44/49(89.8%) cases are positive to HPU primer. This agrees with reports by Espinoza *et al.*, (2011)

ureA gene is housekeeping gene and is needed with others genes for urease enzyme activity (Wen *et al*., 2011). The present study showed positive to *ureA* gene with high percentage up to 89.8%, this makes this gene valuable in detecting this bacterium rather than 16S *rRNA* gene even with high sensitivity as this gene shared by many bacteria, and in comparison to *ureC* gene in present study where it gives low percent (Fig. 7). This comes in contrary to previous study (Espinoza *et al.*, 2011) that had been demonstrated that the amplification of the *ureA* gene (86.36%) was lower than that of the *glmM* (*ureC*) gene (100%); this may be due to polymorphisms that exist within the *ureC* gene (Courcoux *et al.*, 1990).

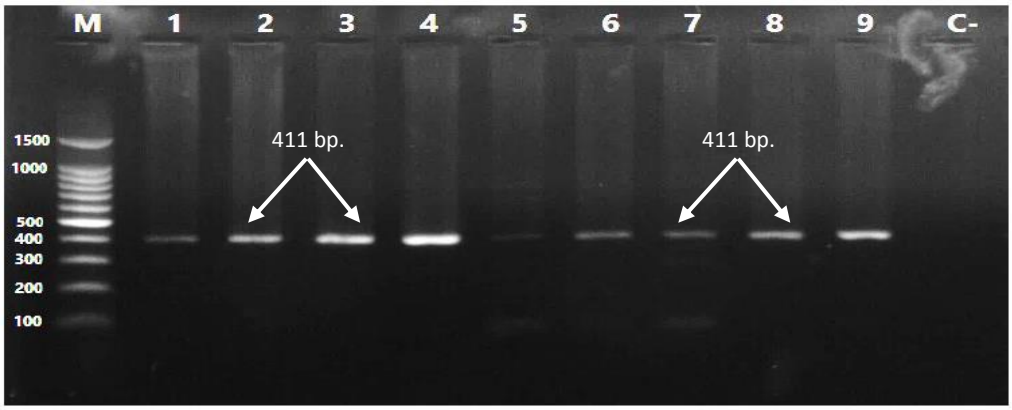

Figure 6: Gel electrophoresis of PCR product of *ureA* gene (411 bp.). 1.5% agarose gel at 7volt /cm for 1 hour. Lane 1-9: positive for *ureA* gene, C-negative control, M: 100-bp DNA marker.

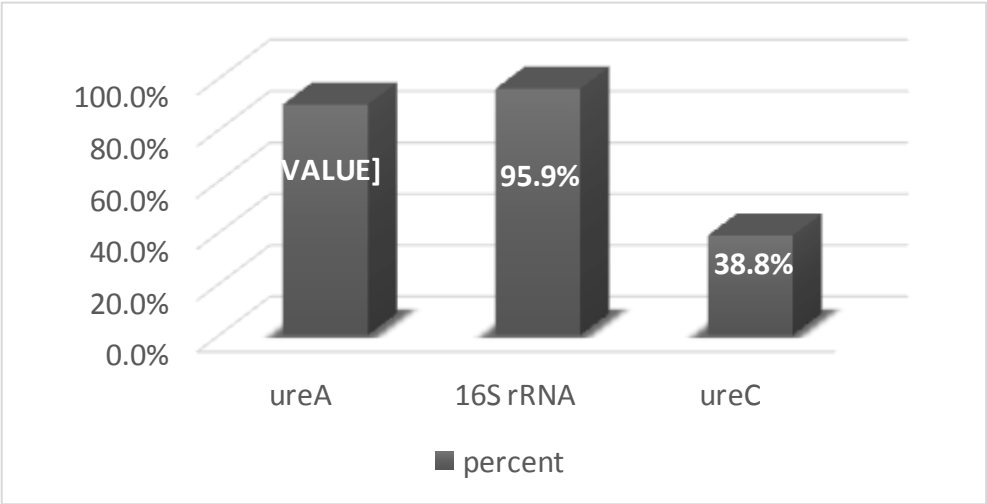

Figure 7: the frequency of *ureA*, 16S *rRNA* and *ureC* genes in positive samples to *H. pylori*

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