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Molecular Detection of *Helicobacter pylori* by Real Time- PCR in Dyspeptic Patients

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Abstract:

Helicobacter pylori is one of the most common infection 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 diseases. In the present study, we used RT-PCR for rapid detection of *H. pylori* infection in peptic ulcer patient, the determination of CagA gene directly from gastric biopsy specimens and determining relationship between gastric cancer and *H. pylori*. Gastric biopsy were collected from 54 patients with disorders in digestive system from AL-Hussein teaching hospital. The results of current study showed that the presence of 16SrRNA for *H. pylori* was 21(38.9%), 15 of 24(62.5%) were males and 6 of 30(20.0%) were females, whereas the percentage of CagA gene was 18(33.3%), 12 of 24 (50%) were males and 6 of 30 (20%) were females by using Real time quantitative PCR in 54 biopsy specimens. These result revealed a significant difference between males and females. Our study indicated that CagA gene RT-PCR is the most specific for the detection of *H. pylori* in gastric biopsy specimens in patients with gastric cancer compared with 16SrRNA gene . There were significant differences ($p \leq 0.05$) in CagA positive rate, among different diseases. In this study the presence of *H. pylori* in patients with Gastric cancer and peptic ulcer has been demonstrated by using RT-PCR method to support the hypothesis that *H. pylori* has a place in etiology of gastric cancer.

Keywords: *H. pylori*, Real time-PCR , Peptic ulcer, Gastric cancer.

1. Introduction:

H. pylori, is microareophilic bacterium, a gram negative and spiral shaped, the bacterium can appear ordinarily in the stomach, in 1982 *H. pylori* discovered by Australian scientists Barry Marshall and Robin Warren, who isolate it from more than 80% of persons with chronic gastritis and gastric ulcer (Blaser,2006). *H. pylori* is able to colonize and persist in the mucus layer of the human stomach. *H. pylori* is 2 to 4 µm in length and usually curved or spiral shaped with 2 to 6 unipolar sheathed flagella of approximately 3 µm in length which allow rapid motility through the viscous mucous layer, coccoid form occur after prolonged culture or antibiotic treatment (Kusters *et al* .,2006). *H. pylori* is the major cause of peptic ulcer, gastritis and gastric mucosa-associated lymphoid tissue (MALT) and gastric lymphoma (Atherton, 2006). The mechanism of *H.pylori* pathogenic effect is uncertain but is believed to be associated to host bacterial interactions started by virulence genes, and it possible that these effects are enhanced by invasiveness of the bacterium (Elitsur and Yahav., 2005; Necchi *et al*., 2007). *H. pylori* released an enzyme called urease which converts urea to ammonia and CO2, this production of ammonia neutralizes the acidity of the stomach, that enable *H. pylori* to survive in the harsh acidic environment of the stomach and making it more hospitable for the bacterium (Atherton, 2006; Kusters *et al*.,2006). *H. pylori* disparity in virulence, two of sequenced strains have an squint 40-kb long Cag pathogenicity island (a generic gene sequence thinks responsible for pathogenesis) that contains over 40 genes (Baldwin *et al*.,2007). A portion of *H. pylori* strains became more virulent by gaining the ability to produce and secrete a protein called cytotoxin-associated gene (CagA) (Fischer, 2011). Gastric cancer is the most common

malignancy and the second major cause of cancer-associated deaths, accounting for 10% of total cancer deaths worldwide (Parkin, 2004). The spaciously majority of gastric cancer are adenocarcinomas, gastric cancer is also characterized by large geographical variations in its incidence and indeed more than half of the total gastric cancer are in East Asian countries such as Japan, south Korea and China (Correa,1988; Shanks and EL-omar,2009). The association of *H. pylori* with gastric cancer has received a great deal of attention and has been thoroughly studies. The International agency for research and the world health organization revealed that *H. pylori* is a group 1 human carcinogen for gastric cancer in 1994 (Franceschi *et al*., 2014). As the clinical outcomes caused by *H.pylori* infection are considered to be related with a complex combination of host susceptibility, environmental factors and bacterial isolates (Delahay and Ruggem, 2012). This study was designed to investigate these correlations.

2.Materials and Methods:

2.1. Sample collection:

Fifty four tissue samples were collected from patients with peptic ulcer disease at period from July 2016 till March 2017 with age range (5-70) years. The samples were divided into children, young and older according to age. Samples were collected from AL-Hussein hospital Digestive system consultation department (DSCD) in Thi-qar province. Before collection of samples, an information sheet was prepared and designed according to some criteria, which including different information like the gender, age, smoking and the history of patient's disease (if the patient had cancer previously, abdominal pain, dyspepsia , weight loss and bleeding per rectum). Upper gastrointestinal tract diseases patients were diagnosed clinically and the disease was evaluated by specialist physicians, patients referred to the Esophago Gastroduodeno Scope Unit for upper endoscopy at AL-Hussein hospital (DSCD) Thi-qar province. Three tissue biopsies were obtained from antrim and body gastric. specimens was preserved immediately at −20°C for molecular analysis.

2.2.Isolation of genomic DNA from gastric biopsy:

With the using of a commercial purification system (The DNA was extracted from the biopsy by Promega Wizard® Genomic DNA Purification Kit) DNA was isolated according to the manufacture's instruction for DNA purification from biopsy. Purified DNA molecules were stored at -80 ˚C, after estimation of DNA concentration and purity, The extracted genomic DNA from biopsy samples was checked by using Nanodrop spectrophotometer (THERMO.USA), that check and measurement the purity of DNA through reading the absorbance in at (260 /280 nm).

2.3. Real-Time PCR:

Real-Time PCR syber green was performed for rapid detection of *H.pylori* according to method described by (Ozyurt *et al*., 2009). *H. pylori* primers were designed in this study by using the complete sequence of *H. pylori* 16S rRNA gene and CagA gene from NCBI Gene-Bank data base and Primer3 plus online provided by (Bioneer company, Korea) as in the following table (1):

Table 1: Primers were used in Real Time PCR

Primer		Sequence	Amplicon
16S RNA gene	F	TGCAAGTCGAACGATGAAGC	86bp
	R	TAAGAGGCACATGACCTATGCG	
CagA primer	F	TGCACAACAACCACAAACCG	108bp
	R	TCAGGATCGTATGAAGCGACAG	

Real-Time PCR technique was performed for detection of *H. pylori* based on amplification of the two detection genes (16S rRNA gene and cagA gene).

2.3.1.Real-Time PCR master mix preparation:

qPCR master mix was prepared by using (**GoTaq®qPCR Master mix**) and this master mix was done according to company instructions as in table (2):

Table 2: Component of reaction mixture in Real Time PCR

PCR Master mix	Volume
DNA template	5µL
16S rRNA gene or CagA gene Forward primer (10pmol)	1µL
16S rRNA gene or CagA gene Reverse primer (10pmol)	1µL
2x Green star Mater mix	10 µL
PCR water	8 µL
Total volume	25µL

After that, these PCR master mix component that mentioned in table(2) above transferred into Exispin vortex centrifuge at 3000rpm for 3 minutes. Then placed in Real-time PCR Thermocycler (BioRad . USA).

2.3.2. Real-Time PCR Thermocycler conditions:

Real-Time PCR thermocycler conditions was set according to primer annealing temperature and qPCR TaqMan kit instructions by Biorad Real-Time PCR thermocycler system as in the following table (3):

Table 3: qPCR thermocycler conditions

Step	Condition	Cycle
Pre-Denaturation	95 °C 5 min	1
Denaturation	95 °C 20 sec	45
Annealing/Extension	60 °C 30 sec	
Detection (Scan)		

2.4.Statistical analysis:

Data were analyzed with chi-square and P value < 0.05 was considered statistically significant.

3. Results:

The present study carried out on 54 patients with peptic ulcer from Al-Hussein Teaching Hospital that included in this study, 24 male patients and 30 female patients, to elucidate *H. pylori* infection by using Real Time Polymerase Chain Reaction(RT-PCR). Results presented in this study showed that overall the prevalence of 16SrRNA gene of *H. pylori* were 21of 54(38.9%) among those 15 of 24(62.5%) were males and 6 of 30(20.0%) were females by using RT-PCR technique while the overall prevalence of CagA gene were 18 of 54(33.3%) among those 12 of 24 (50%) were males and 6 of 30 (20%) were females by using Real time quantitative PCR. However the results of current study showed significant different at p≤ 0.05 between male and female. as shown in table(4).

Table (4): Distribution of peptic ulcer patients according to gender

Gender	RT-PCR							
	16SrRNA				Cag A gene			
	positive		negative		positive		negative	
	No	%	No	%	No	%	No	%
Male(24)	15	62.5	9	37.5	12	50	12	50
Female(33)	6	20.0	24	80.0	6	20	24	80
Total	21	38.9%	33	61.1	18	33.3%	36	66.7
Statistical	X^2=10.13, df=1, p=0.001				X^2=5.4 df=1, p=0.02			

Detection of *H. pylori* genes (16SrRNA and CagA gene) by RT-PCR

Besides the clinical investigations by specialist physicians in detection of peptic ulcer, Real-Time PCR (RT-PCR) technique was used to confirm the infection with *H. pylori* by detection of *H. pylori* DNA in the biopsy samples of patients. *H. pylori* DNA was successfully extracted and analyzed by RT-PCR. The study revealed that out of 54 patients only 21 (38.9%) showed positive with 16SrRNA gene of *H. pylori* and only 18 (33.3%) showed positive with CagA gene by RT-PCR. Statistically, no significant difference between 16SrRNA and CagA gene($p<0.05$) as showen in table (4). The positive amplification samples of 16SrRNA and CagA gene which shown cross up the threshold cycle number, where the negative samples show as undetermined by amplification plot as shown in figures (1) and (2) respectively.

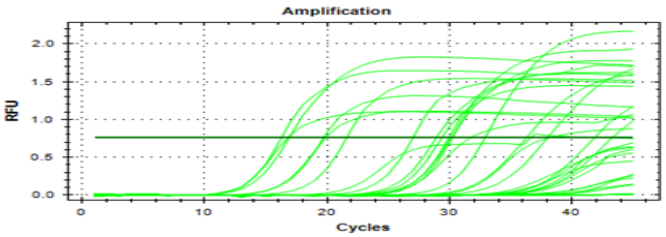

Figure (1): Real-Time PCR amplification plots of 16S rRNA gene in *H. pylori* based on SYBER green DNA binding dye. Where, the positive amplification samples which shown cross up the threshold cycle number.

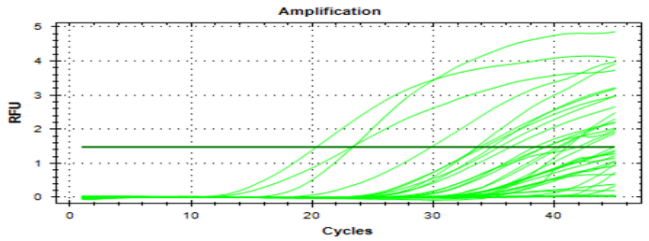

Figure (2): Real-Time PCR amplification plots of cagA gene in *H.pylori* based on SYBER green DNA binding dye. Where, the positive amplification samples which shown cross up the threshold cycle number

Distribution of patients according to clinical investigation

Depending on clinical diagnosis the patients were suffering from gastric cancer, gastric ulcer, pangastropathy, Lax cardia and Prolapse gastropathy, the current results demonstrated the correlation between

H. pylori and gastric cancer. This results revealed the prevalence of CagA gene of *H. pylori* in all dyspeptic patients by RT-PCR, and the higher value of CagA gene in gastric ulcer and gastric cancer was (63.6% and 50%) respectively, whereas the lower value was (10%) in lax cardia, Whereas the prevalence of 16SrRNA gene of *H. pylori* in all dyspeptic patients by RT-PCR, and the higher value of 16SrRNA gene in patients with Prolapse gastropathy was (55.5%), while the lower value was (20%) in patients with Gastric cancer, However the results of CagA gene in current study showed significant different at $p \leq 0.05$ between patients, whereas no significant different at $p \leq 0.05$ between patients in results of 16SrRNA gene and table (5) explain the details.

Table 5: Distribution of peptic ulcer patients by using RT-PCR according to diseases

Diseases	Test							
	Cag A gene				16SrRNA gene			
	Positive		Negative		Positive		Negative	
	NO	%	NO	%	NO	%	NO	%
Gastric cancer	5	50.0	5	50.0	3	30	7	70
Gastric ulcer	7	63.6	4	36.4	4	36.4	7	63.6
Pangastropathy	3	21.4	11	78.6	5	35.7	9	64.3
Lax cardia	1	10.0	9	90.0	4	40	6	60
Prolapse gastropathy	2	22.2	7	77.8	5	55.6	4	44.4
Total	18		36		21		33	
Statistical	X^2=9.638, df=4, p≤0.05				X^2=1.47,df=4,p≥0.05			

Discussion:

In the present study, we have investigated a group of consecutive dyspeptic patients by endoscopy and gastric biopsy for *H. pylori* infection. The results revealed the percentage of 16SrRNA for *H. pylori* was(38.9%) by using RT-PCR, whereas the percentage of CagA gene for *H. pylori* was(33.3%) , this result was lower than other previous studies by Kalaf *et al*.,(2013) who recorded the percentage of 16SrRNA and CagA gene was 91.17% and 39.21% respectively. Among 54 dyspeptic patients, 18 (33.3%) were positive for cagA gene. There were significant differences ($P \leq 0.05$) in cagA positive rate, among different type of gastric diseases under investigation, this further substantiate the role of cag A as a marker for increased virulence of *H. pylori* (Table 5). The result of present study showed presence of Cag A gene in patients with gastric ulcer was 63.6%, this results was corresponding with other previous study by Faundez *et al*., (2002) who found the percentage of Cag A gene in patient with peptic ulcer was 60%. The result of present study evaluate the percentage of Cag A gene in gastric cancer patients was 50%, this result was match with other study by Satomi *et al*.,(2006). The rate differs from very high in East Asian countries to low from many Middle Eastern countries. For example, 94% in Malaysia (24), 90% in China (25). And among *H. pylori* strains isolated in the Middle East , 60.8% in Saudi Arabia, 53% in Kuwait (26), 92% in Turkey (21) . Low prevalence of strains with cag A positive in Iraqi patients might be reasons for low incidence of gastric cancer in Iraq. Assays with RT-PCR 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 (Kalaf et al. ,2013).

The percentage of 16SrRNA for *H. pylori* in the present study among males was 15 of 24 (62.5%) are higher than those in females 6 of 33 (20.0%) within dyspeptic patients, whereas the percentage of CagA gene for *H. pylori* in the present study among males was 12 of 24 (50%) are higher than those in females 6 of 33 (20.0%) within dyspeptic patients. The results of current study was matched with other studies in Iraq by Amer *et al*.,(2014) and Ihsan *et al*.,(2014) which found the percentage of *H.pylori* in infected males was more than those in infected females. While this results was different from other studies by (Módena et al., 2007) who found the percentage of *H .pylori* in infected males was lower than those in infected females. while some other studies have noticed no such relation to gender(Petrovic *et al*., 2011; Alazmi *et al*., 2010). The differences in socioeconomic conditions in the peptic ulcer patients and the type of males jobs can explain this discrepancy.

Reference:

Alazmi, WM.; Siddique, I.; Alateeqi, N. and Al-Nakib, B. (2010). Prevalence of *Helicobacter pylori* infection among new outpatients with dyspepsia in Kuwait. BMC Gastroenterol. 10:14.

Al-Jobori, M.; Al-Ouqaili, M. and Abdullah, E. (2011). 21. Detection of 16S rRNA gene of *Helicobacter pylori* in patients with peptic ulcer and gastric carcinoma: molecular and

bacteriological study.Egypt. Acad. J. Biolog. Sci. 3: 95- 104.

Atherton, J. (2006) . The Pathogenesis of *H. pylori* Induced Gastro-Duodenal Diseases. Annual Review of Pathology: Mechanisms of Disease, 1, 63-96.

Baldwin, DN.; Shepherd, B.; Kraemer, P.; Hall, MK.; Sycuro, LK.; Pinto-Santini, DM. and Salama, NR. (2007). "Identification of *H. pylori* Genes That Contribute to Stomach Colonization". Infect Immun. 75 (2): 1005–16.

Blaser, MJ. (2006). "Who are we? Indigenous microbes and the ecology of human diseases" (PDF). EMBO Reports. 7 (10): 956–60.

Correa, P. (1988). A human model of gastric carcinogenesis. Cancer Res. 48, 3554–3560.

Delahay, RM . and Rugge, M. (2012). Pathogenesis of *Helicobacter pylori* infection. Helicobacter. 2012;17 Suppl 1:9–15.

Faundez, G., Troncoso, M., and Figueroa, G. (2002). cagA and vacA in strains of *Helicobacter pylori* from ulcer and non- ulcerative dyspepsia patients, *4*, 4–7.

Fischer, W. (2011). Assembly and molecular mode of action of the *Helicobacter pylori* Cag type IV secretion apparatus. FEBS J. 278, 1203–1212.

Franceschi, F.; Tortora, A.; Gasbarrini, G. and Gasbarrini, A.(2014). *Helicobacter pylori* and extragastric diseases. *Helicobacter*. 2014;19 Suppl 1:52–8.

Kalaf, A. E.; Yassen, Y.N; AL-Khafaji, Z. M; A'Rif, G. L. and Abass, B. A. (2013). Multiplex PCR assay for detection of *Helicobacter pylori* isolated from Iraqi dyspeptic patients.

Kusters, JG.; van Vliet, AH. and Kuipers, EJ.(2006). Pathogenesis of *Helicobacter pylori* infection. Clinical Microbiology Reviews ; 19(3):449–490.

Módena, J. L. P.; Acrani, G. O.; Micas, A. F. D.; Castro, M. De; Silveira, W. D. Da; Módena, J. L. P.and Brocchi, M. (2007). Correlation between Helicobacter pylori infection, gastric diseases and life habits among patients treated at a university hospital in Southeast Brazil. The Brazilian Journal of Infectious Diseases : An Official Publication of the Brazilian Society of Infectious Diseases, 11(1), 89–95.

Mohamed, R.; Hanafiah, A.; Rose, I.; Manaf, M.; Ab24. dullah, S.; Sagap , I.; Belkum, A. and Yaacob, J.(2009). *Helicobacter pylori* cagA gene variants in Malaysians of different ethnicity. Eur. J. Clin .Microbiol. Infect. Dis. 28: 865–9.

Mohammed, M. (2012). Relationship between *Helicobacter pylori* 26. virulence genes and clinical outcomes in Saudi patients. J. Korean Med. Sci. 27: 190-3.

Ozyurt, M.; Gungor, A.; Ergunay, K.; Cekin, E.; Erkul, E. and Haznedaroglu, T.(2009). Real-time PCR detection of *H. pylori* and virulence-associated cagA in nasal polyps and laryngeal disorders. Otolaryngol Head Neck Surg; 141:131-5.

Parkin, D.M. (2004). International variation. Oncogene 23, 6329–6340. Peek, R.M., Jr., and Blaser, M.J. (2002). *Helicobacter pylori* and gastrointestinal tract adenocarcinomas. Nat. Rev. Cancer 2, 28–37.

Petrovic, M.; Artiko, V.and Novosel, S. (2011). Relationship between *H. pylori* infection estimated by 14C-urea breath test and gender, blood groups and Rhesus factor. *Hell J Nucl Med*. ;14:21–24.

Satomi, S.; Matsunaga, Y, A.; Masaki, S. R., and Inagaki, T. (2006). Relationship between the diversity of the cagA gene of *H. pylori* and gastric cancer in Okinawa , Japan, 668–673.

Shanks, A.M., and El-Omar, E.M. (2009). *Helicobacter pylori* infection, host genetics and gastric cancer. J. Dig. Dis. 10, 157–164.

Necchi, V.; Candusso, M. E. and Tava, F. (2007). Intracellular, intercellular, and stromal invasion of gastric mucosa, preneoplastic lesions, and cancer by *Helicobacter pylori*, Gastroenterology, vol. 132, no. 3, pp. 1009–1023.

Wang, X.; Terry, H.; Cheng, J.; Wu, L. and Hu, S. (2011). 25. Interactions between CagA and smoking in gastric cancer. World J. Gastroenterol.17: 3330-4.

Weeks, D.; Eskandari, S.; Scott, D. and Sachs, G. (2000). - A H+-gated urea channel: the link between *Helicobacter pylori* urease and gastric colonization. Sci. 287 (5452):482–485.

Elitsur, Y. and Yahav, J. (2005). *Helicobacter pylori* infection in pediatrics, *Helicobacter*, Supplement, vol. 10(1): 47–53.