**ORIGINAL ARTICLE**

**Helicobacter pylori** CagA antigen detection of gastric infected patients and its association to severity of disease in Iraq

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**ABSTRACT**

**Objective:** Helicobacter pylori (H. pylori) is associated with various upper gastrointestinal tract disorders. Many studies have measured anti-cytotoxin-associated gene A (CagA) antibody concentration as signs of CagA antigen presence, but our study aimed to find an association between the CagA antigen and the severity of H. pylori gastric infections in Iraqi patients by measuring the CagA antigen itself instead of anti-CagA antibody.

**Methods:** Ninety three samples were selected of the mentioned number included antral biopsies and blood from patient with age range of 11 to 85 years. Rapid urease test (RUT) and gene polymerase chain reaction (PCR) of phosphoglucosamine mutase (glmM) used for H. pylori detection. cagA gene detection have been used to detecting whether there were association between symptoms and cagA gene presence. Serum CagA antigen level have been measured by ELISA technique.

**Results:** Serum CagA antigen detection indicated a significant difference between asymptomatic and symptomatic groups (p≤0.000). There were strong association between CagA antigen concentration and severity of disease. The significant difference between gastric ulcer and duodenal ulcer, gastric ulcer and gastritis and duodenal ulcer and gastritis was (p≤0.006), (p≤0.000) and (p≤0.01), respectively. Although there was no significant difference between males and females of asymptomatic group, in symptomatic patients the mean concentration was significantly higher (p≤0.002) in females than in male.

**Conclusion:** This study show strong association between presence of cagA gene and its product and peptic symptoms of Iraqi patients.

**INTRODUCTION**

Peptic injury has been an unquestionable disease of the twentieth century. It may include gastritis of incidence of a sore on the mucus lining of the stomach or duodenum¹. Most common severe peptic infections are Gastric ulcer, lining damage of stomach, and duodenal ulcer, almost due to excessive acid secretion of the stomach glands. However, H. pylori is one of the most important microbial factors play role in peptic
infections\textsuperscript{2}. \textit{H. pylori} is Gram-negative bacterium with spiral appearance with S-shape includes 1 to 3 turns and rounded ends. It is about 2.5 to 3.5 μm in length, a diameter of 0.5 to 1μm and 1 to 2μm periodicity and has smooth surface. It contains about 6 polar sheathed flagella\textsuperscript{3}. The flagella have size about 3μm in length and distinctive terminal bulb\textsuperscript{3}. \textit{H. pylori} has high mobility in viscous solutions, and its flagella play an important role in this motility\textsuperscript{4}. It can grow in microaerophilic conditions\textsuperscript{5} and inhabit mucus layer of the gastric epithelium of many mammals and birds\textsuperscript{6}.

Cytotoxin-associated gene A (CagA) antigen, the major responsible for pathological feature of \textit{H. pylori} infection, is a protein with molecular weight of about 120-145 kDa and its coding gene included within cag Pathogenicity Island (cagPAI). It is also considered as the first bacterial onco-protein which nomimates the \textit{H. pylori}-mediated adenocarcinoma as the second most lethal cancer type in the world\textsuperscript{7}. CagPAI is the region of about 40-kb of \textit{H. pylori} chromosome includes the mentioned gene and approximately 29 genes coding for a type IV secretion system (T4SS) dependent secretion of CagA toxin\textsuperscript{8}. CagA is the only protein known to be secreted by the Cag T4SS in \textit{H. pylori}. One of the main characteristics of CagA is its capability to interacting with the host cell kinase, undergoing tyrosine phosphorylation modifications\textsuperscript{9}. The tyrosine phosphorylation site of CagA involves conserved arrangement of amino acid motifs located in the C-terminal region of the protein and consists of Glutamic Acid (Glu) -Proline (Pro) -Isoleucine (Ile) -Tyrosine (Tyr) -Alanine (Ala) (together called EPIYA)\textsuperscript{9}. Geographically and on the basis of CagA- positive strains of \textit{H. pylori} and amino acid sequences that flank them, four worldwide distributed characteristic types of EPIYA motifs, A,B,C and EPIYA-D, have been identified. The first two of them, --A and --B, generally are contained in all CagA-positive strains but EPIYA-C is mainly found in strains that harvested in western countries such as USA, Europe and few Asian countries such as India and Malaysia (Western type CagA), while EPIYA-D is mainly found in the Eastern strains countries (Far East Asian regions such as Korea, China, Japan) (Eastern type CagA)\textsuperscript{10}. The multiplicity of EPIYA motif numbers at the C-terminal of CagA can vary due to repetitive amino acids flanking these motifs. So this determines the EPIYA motifs variability number that carried by different CagAs\textsuperscript{11} and indeed the variance in pathogenicity of different CagA-positive strains\textsuperscript{12}. For instance, transfection of human gastric epithelium in vitro revealed that strains with ABCCC type-CagA will trigger significant numerous genes transcription involved in gastric carcinogenesis compared to the strains with ABC-type CagA\textsuperscript{13}. In Iraq, \textit{H. pylori} strains possess virulence factors comparable to those in Western countries\textsuperscript{14}. For CagA insertion into the gastric epithelial cells (GECs), \textit{H. pylori} form a syringe-like pilus structure. It could be done by some of T4SS proteins, CagL, CagY and CagA. The latter by itself binding to integrin β1on the host cell surface, passed across the host cell membrane. Also the importance of interaction between CagA and phosphatidylinerine has been shown for the injecting process\textsuperscript{15}. After delivery and tyrosine phosphorylation at motifs containing the amino acid sequence EPIYA of CagA, it will deregulate intracellular signaling pathways and thereby initiates pathogenesis\textsuperscript{16,17}.

Phosphorylated CagA has the ability to interact with the sarcoma (Src) Homology Phosphatase 2 (SHP-2) tyrosine phosphatase, and make it functionally active, trigger a host cell morphological change to a actively motile phenotype known as the ‘hummingbird phenotype’\textsuperscript{18}. This cell-scattering phenotype participates in different aspects of carcinogenesis, including metastasis. CagA is also a very antigenic protein, associated with a prominent inflammatory response due to eliciting interleukin-8 (IL-8) production\textsuperscript{9}. CagA/SHP-2 interaction is also cause deregulation of cell-cycle and increase cellular proliferation\textsuperscript{19}. It has been found that exosomes released from CagA expressing gastric epithelial cells may reach the circulation, carrying CagA to distant tissues and organs in its active phosphorylated form. So, CagA-containing exosomes may be implicated in the expansion of extragastric disorders related with cagA-positive \textit{H. pylori} infection\textsuperscript{19}. However, many studies have measured anti-CagA antibody concentration as signs of CagA antigen presence, but this study aimed to find an association between the CagA antigen and the severity of \textit{H. pylori} related gastric infections in Iraqi patients by measuring the CagA antigen itself instead of anti-CagA antibody.

**MATERIALS AND METHODS**

**Sample collection:** In this study two types of measurements were taken into consideration: comparing between intact persons (controls) and infected individuals and also among patients themselves, those with normal gastroduodenoscopic finding and those with abnormal gastroduodenoscopic finding. Due to the presence of two types of \textit{H. pylori}, cagA positive and cagA negative, we had must collect biopsies of two types of patients as probably associated with the role of this gene to intensity of peptic infections; biopsies from patients with normal finding of gastroduodenoscopy called positive asymptomatic and from those with abnormal finding of gastroduodenoscopy (in this study gastritis (inflammation, friability, edema, punctuate hemorrhage and nodularity), gastric ulcer and duodenal ulcers) called positive symptomatic. And there were also biopsies taken from suspected persons also with similar symptoms (reflux, abdominal pain and dyspepsia), but have any infection, called negative control\textsuperscript{20}. So, there are two types of control: negative for either person is infected with \textit{H. pylori} or not and asymptomatic for the probably absence or presence of cagA gene in positive patients.

Patients samples were collected in Kerbala (Al Hussein Educational hospital, Al-Kafeel hospital) and Babylon (Merjan hospital) governorates between 26-Desember-
2016 and 26-march-2017. Informed consent was obtained from all patients. Patients included males (45) and females (48). Their ages ranged from 11 to 85 with a median of 35 years and age mean of 36.87 year. The gastroduodenoscopy were described by the gastroenterologist. Patients with malignancies, gastric surgery, Cardiovascular and autoimmune disease and who take antibiotic, proton pump inhibitors such as Omeprazole and non-steroidal anti-inflammatory drugs such as aspirin, diclofenac, ibuprofen etc. have been excluded. We also excluded the patients with abnormal gastroduodenoscopy finding but negative RUT results.

Two biopsies from antral region of stomach had taken and have used for RUT and Polymerase chain reaction (PCR). Also, blood sample collected for antigen detection in serum by Enzyme-linked immunosorbent assay (ELISA).

**Rapid urease test:** Urea agar base, for RUT, prepared based on container instructions and then 40% urea is filtered with micro filter paper, added to medium and mixed well, then poured into Eppendorf tube (1.5 ml) before been solidified. Rapid Urease Test was performed in the endoscopy room immediately by placing antral biopsy into Eppendorf tube contain solidified 1.5 ml of urea agar base media and waited to about 30 min. however color changing take time from a few minutes to 24 hrs. Color changes from yellow to pink indicated positive result and unchanged color regarded as a negative result.

**DNA extraction:** In vitro Each frozen biopsy specimen was thawed, crushed; genomic DNA was then extracted directly using Favorgen tissue genomic DNA extraction mini kit-USA, as described by the manufacturer. The concentration and purity of DNA samples were determined by using Nanodrop Instrument (A micro volume measurement platform).

**Molecular (PCR) detection:** Monoplex PCR protocol has been used to investigating each of Phosphoglucomase mutase gene (glmM) and cagA genes using primers produced by Primer company-China. Table 1 shows Oligonucleotide sequence of primers and PCR product sizes of glmM and cagA genes.

Table 1: Oligonucleotide sequence of primers and PCR product sizes

<table>
<thead>
<tr>
<th>Amplified Gene</th>
<th>primer</th>
<th>Sequence (5'-3')</th>
<th>PCR Product Size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>glmM</td>
<td>glmM-F</td>
<td>AGCTTTTAA GGTTA GGGTTT</td>
<td>294</td>
<td></td>
</tr>
<tr>
<td></td>
<td>glmM-R</td>
<td>AAGCTTACTTTAACA CTAACGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cagA</td>
<td>cagA-F</td>
<td>GATAACAGCAGGCTT TGAAG</td>
<td>349</td>
<td></td>
</tr>
<tr>
<td></td>
<td>cagA-R</td>
<td>CTGAAAAGGTTT GCAGA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

https://www.ncbi.nlm.nih.gov/tools/ primer-blast/index.cgi?LINK_LOC=BlastHome online website used for Finding primers specific to our PCR templates. For glmM gene amplification, each PCR tube has contained 12.5μl of master mix (Biolabs- England), 2μl of forward and 2μl revers primers, 5 μl of template DNA and 3.5 μl of deionised water (total reaction volume: 25 μl). For cagA gene amplification, each PCR tube has contained 12.5μl of master mix (Biolabs- England), 2μl of forward and 2μl revers primers, 3 μl of template DNA and 7.5 μl of deionised water (total reaction volume: 25 μl).

**Agarose Gel Electrophoresis:** Amplified genes were analyzed by 2% agarose gel electrophoresis (w/v) using TBE 0.5X.

**Enzyme-linked immunosorbent assay Protocol:** The serum CagA antigen level assayed by the ELISA method using a commercial sandwich ELISA kit manufactured by MyBioSource/ USA.

**Statistical Analysis:** Statistical analysis was done by using the software statistical package for social sciences (SPSS; version 18). The results were expressed as mean ± standard deviations (Mean ± S.D). Statistical analysis for the significance of differences of the quantitative data was done by using ANOVA test for single factor means, while, the Pearson's coefficient was used for the determination of the correlation between CagA antigen level and severity of peptic infections. P-values ≤ 0.05 were considered statistically significant.

**RESULTS**

Patient genders, numbers, ages range and RUT results have been shown in Table 2.

<table>
<thead>
<tr>
<th>Variable</th>
<th>No.</th>
<th>Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10 – 20</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>21 – 40</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>41 – 85</td>
<td>6</td>
</tr>
<tr>
<td>females</td>
<td>Asymptomatic</td>
<td>10 – 20</td>
</tr>
<tr>
<td></td>
<td>21 – 40</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>41 – 85</td>
<td>5</td>
</tr>
<tr>
<td>Symptomatic</td>
<td>10 – 20</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>21 – 40</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>41 – 85</td>
<td>6</td>
</tr>
<tr>
<td>Total</td>
<td>48</td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td>Asymptomatic</td>
<td>10 – 20</td>
</tr>
<tr>
<td></td>
<td>21 – 40</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>41 – 85</td>
<td>3</td>
</tr>
<tr>
<td>Symptomatic</td>
<td>10 – 20</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>21 – 40</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>41 – 85</td>
<td>10</td>
</tr>
<tr>
<td>Total</td>
<td>45</td>
<td></td>
</tr>
</tbody>
</table>

Second and final step of *H. pylori* detection has been accomplished by appearance of glmM gene product of 294 bp. The glmM gene is highly conserved and could be used to *H. pylori* Identification in gastric biopsies. One of the features of the glmM gene using in *H. pylori* Identification in the gastric biopsies is its absolute sensitivity and specificity, since it presents detection rate of 10 to 100 *H. pylori* cells which is even better than histopathology. All samples of asymptomatic and symptomatic patients were contained the gene. Figure 1 shows the PCR results of glmM gene. The third step of work was to detect whether samples contain cagA gene or not. This step was also applied on
all three groups of samples. Figure 2 shows a product of 349 bp in size as the PCR results.

The results of PCR technique were shown a strong association between the cagA gene presence and symptoms (Table 3).

**Table 3: Distribution of cagA Gene in asymptomatic and symptomatic patient.**

<table>
<thead>
<tr>
<th>Groups</th>
<th>No.</th>
<th>Positive</th>
<th>Percentage%</th>
<th>Negative</th>
<th>Percentage%</th>
</tr>
</thead>
<tbody>
<tr>
<td>asymptomatic</td>
<td>23</td>
<td>1</td>
<td>4.35</td>
<td>22</td>
<td>95.65</td>
</tr>
<tr>
<td>symptomatic</td>
<td>44</td>
<td>41</td>
<td>93.18</td>
<td>3</td>
<td>6.82</td>
</tr>
</tbody>
</table>

The results of serum CagA antigen detection showed a significant difference between asymptomatic and symptomatic groups (p≤0.000). In symptomatic patients the mean concentration was 540.15 ± 142.54 pg/ml while in asymptomatic patients it was 212 ± 193.8 pg/ml Figure 3.

By comparing a presence of the cagA gene and its product with gastroendoscopic finding it has found that there is an intimate link between CagA antigen and intensity of disease and with significant difference. Toxin serum levels were higher in gastric ulcers (765.83 ± 120.8pg/ml) than duodenal ulcers (574 ± 112.2 pg/ml) and gastritis infections (479.71 ± 99.6 pg/ml) Figure 4. The significant difference between gastritis and duodenal ulcer, duodenal ulcer and gastric ulcer and gastritis and gastric ulcer was (p≤0.01), (p≤0.006) and (p≤0.000) respectively.

Although there was no significant difference between males and females of asymptomatic group, the mean concentration was higher in females (218.35 ± 199.92 pg/ml) than males (202.11 ± 195.35 pg/ml; p≥0.8). However, in symptomatic patients the mean concentration was significantly higher (p≤0.002) in females (611.05 ± 109.01 pg/ml) compared to males (486.28 ± 138.54 pg/ml). Comparison within ages shows conflicting results in both asymptomatic and symptomatic groups.

**Discussion**

To find the relation between cagA gene and symptoms of disease, its product must be measured in serum of symptomatic and asymptomatic patients. In contrast to approximately all studies that had detected anti-CagA antibody in serum of patients as the evidence for the CagA toxin presence, in the current study CagA antigen detection technique rather than CagA antibody detection technique was used by sandwich ELISA for detection of CagA protein in serum of patients. So this step could be regarded as the novel method, at least in our country. Franceschi et al. (2009) showed that there is mimicry between CagA antigen and antigens found in the atherosclerotic plaque occurred in tibial arteries. So in H. pylori infected patients, intensive anti-CagA response to the CagA antigen may also include the protein localized in coronary atherosclerotic plaques and cause coronary instability. This is due to mimicry between these proteins and CagA antigen31. On the other hand, anti-CagA immunoglobulin secreted against the CagA is


