

## ***Helicobacter pylori* cagA gene Polymorphism in Patients with Gastroduodenal Diseases**

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### **Abstract**

*Helicobacter pylori* is a genetically diverse bacterial pathogen and its *CagA* gene is a major virulence factor that plays an important role in gastroduodenal pathologies. The biological function of *cagA* depends on tyrosine phosphorylation within the EPIYA (Glutamate-Proline-Isoleucine-Tyrosine-Alanine) motifs at the C-terminal region of the protein. This region may undergo polymorphism to give different types of EPIYA motifs. EPIYA motif diversity may provide a useful tool for prediction of *H. pylori* pathogenic activity and accurate determination of number and type of *cagA* EPIYA motifs could identify the virulent *H. pylori*. The aim of this study was to detect *H. pylori cagA* gene and its polymorphism in endoscopic gastroduodenal biopsy specimen from patients with gastroduodenal diseases in Bangladesh. This cross sectional study was carried out in the Department of Microbiology & Immunology, Bangabandhu Sheikh Mujib Medical University and Center for Advanced Research in Sciences, University of Dhaka during the period from March 2014 to February 2015. Gastric biopsies were collected from 78 patients with gastritis, duodenal ulcer, gastric ulcer and gastric carcinoma. *H. pylori* was identified by rapid urease test and *ureC* gene PCR. Presence of *cagA* gene and number and pattern of *cagA* EPIYA motif were determined by PCR. DNA sequencing was carried out to confirm the PCR detection method of *cagA* EPIYA motif and to analyse their peptide sequence. Among 31(39.7%) *H. pylori* positive cases, 19 (61.3%) were *cagA* gene positive in 11(55%) gastritis, 4(66.7%) duodenal ulcer, 2(66.7%) gastric ulcer and 2(100%) gastric carcinoma. A significant association was found between *cagA* gene and duodenal ulcer ( $p<0.05$ ). EPIYA motif of all *H. pylori cagA* positive cases showed Western type *cagA* EPIYA ABC. No East Asian EPIYA ABD motif was found. Majority of gastroduodenal cases (57.9%) had 3 copies of EPIYA (ABC type), 26.3% had 4 copies (ABCC type) while remaining 10.5% had AC and 5.2% AB type EPIYA motif. EPIYA ABC was found in 75% of duodenal ulcer followed by 54.5% of gastritis and 50% of both gastric ulcer and gastric carcinoma patients. EPIYA ABCC motif was found in 50% of gastric ulcer and gastric carcinoma patients. Most of the EPIYA motif was EPIYA ABC and some were ABCC which has the risk of developing gastric carcinoma.

**Keywords:** EPIYA (Glutamate-Proline- Isoleucine-Tyrosine- Alanine), polymorphism, PCR

### **Introduction**

*Helicobacter pylori* is a spiral shaped microaerophilic bacterium that colonizes gastric mucosa of more than half of the world's population and is associated with development of complications such as peptic ulcer disease, gastric carcinoma and mucosa associated

lymphoid tissue lymphoma.<sup>1</sup> The association between *H. pylori* and gastric carcinoma led the World Health Organization to classify it as a class-I carcinogen in 1994. Bangladesh is a developing country and epidemiological studies shown 92% adult population were seropositive for *H. pylori*.<sup>2</sup>

*Helicobacter pylori* strains can be divided into two major types based on their ability to produce a 120–145kDa immune-dominant protein called cytotoxin associated gene A (*CagA*) antigen.<sup>3</sup> *H. pylori* strains possessing the *CagA* gene were linked with an increased risk of developing gastric cancer and peptic ulcer. The risk of developing gastric cancer in *H. pylori* infected *CagA*-positive subjects is six fold higher than that in *CagA*-negative subjects.<sup>4</sup> More than 90% of isolated strains from East Asia including Korea, Japan, and China are known to harbor *cagA*, while 50%-60% of isolated strains from Western countries are positive for it.<sup>5</sup>

The 3'-end region of *cagA* where the tyrosine phosphorylation sites are located are highly polymorphic.<sup>6</sup> Phosphorylation occurs on specific tyrosine residues within repeating penta amino acid Glu-Pro-Ile-Tyr-Ala (EPIYA) motifs, present at the C-terminus of the protein.<sup>7</sup> Four different *CagA* EPIYA motifs, EPIYA-A, -B, -C, and -D have been defined based on the amino acid sequences surrounding the EPIYA residue.<sup>8</sup> *CagA* proteins nearly always possess an EPIYA-A and an EPIYA-B, followed by various number of EPIYA-C repeats in Western-type or EPIYA-D motifs in East Asian type strains.<sup>6,9</sup> It has been suggested that the considerable variation in number of repeating EPIYA-C or -D motifs determines the biological activity of *cagA*.<sup>7</sup>

*CagA* strains possessing multiple number EPIYA C segments predisposes to precancerous lesions and gastric cancer.<sup>7,10</sup> Western type EPIYA ABC pattern present in 100% *H. pylori* strains isolated from patients with various gastroduodenal diseases in India.<sup>11</sup> The phylogenetic analysis of the 5' end of the *cagA* gene indicates that Bangladeshi isolates are more closely related to *H. pylori* isolates from India and are different from isolates of East Asia.<sup>12</sup>

As large number of populations in Bangladesh are seropositive for *H. pylori* so, it is important to know the number and pattern of *cagA* EPIYA motifs for identifying the *H. pylori* infected patients who tend to develop severe gastroduodenal diseases. Therefore, EPIYA motif diversity may provide a useful tool for prediction of *H. pylori* pathogenic activity and accurate determination of number and type of

*cagA* EPIYA motifs could identify the virulent *H. pylori* causing severe Gastroduodenal diseases in Bangladesh. So, this study was designed to detect *H. pylori* *cagA* gene polymorphism in gastric biopsy specimen from patients with gastroduodenal diseases.

## Materials and Methods

Patients with dyspeptic symptoms who underwent endoscopic examination in the Department of Gastroenterology of Bangabandhu Sheikh Mujib Medical University (BSMMU) and Dhaka Medical College Hospital had endoscopic findings of any inflammation, ulcer or growth in gastroduodenal mucosa were enrolled in the study. Six biopsy specimens were collected from each patient: three from the gastric antrum and three from the gastric body.<sup>13</sup> Each specimen from antrum and body was used for rapid urease test, PCR for *ureC* gene and histopathological examination. All procedures were performed in the Department of Microbiology and Immunology, BSMMU, Dhaka during the period of March, 2014 to February, 2015. Patients with history of previous gastric surgery, *H. pylori* eradication therapy or treatment with antibiotics, bismuth containing compounds, H<sub>2</sub>-receptor blockers or proton pump inhibitors within four weeks prior to enrolment were excluded from the study. Based on the endoscopic and histopathological assessments, samples were divided into four groups of gastritis, duodenal ulcer, gastric ulcer and gastric carcinoma. Institutional Review Board, BSMMU and Ethical Review Committee of Dhaka Medical College approved the study protocol and informed consent was obtained from each patient prior to endoscopy and biopsy specimen collection.

**DNA extraction from gastric tissues:** DNA from gastric tissues was extracted by using the QIAamp (QIAGEN) DNA Mini Kit according to the manufacturer's instruction.

**PCR assay:** For confirming the presence of *H. pylori* DNA in gastric tissues, the *ureC* gene was identified by PCR using the primers<sup>14</sup> (forward 5' AAGCTTTTAGGG GTGTTAGGGGTTT3' and reverse 5' AAGCTTACTTTCTAACACTAACG C 3' with a 294-bp size product. For detecting the presence of the *cagA* gene, PCR was performed by using primer pairs: forward (*cagAF*) 5'-

GATAACAGGCAAGCTTTTGAGG-3' and reverse (*cagAR*) 5'-CTGCAAAAGATTGTTTGCAGA -3' with a 349-bp size product.<sup>6</sup> The forward primer *cagA28F* (5' TTCTCAAAGGAGCAATTGGC 3') and reverse primers *cagAP1C* (5'GTCCTGCTTTCTTTTTATTAACCTKAGC 3'), equimolar mixture of *cagAP2CG* (5'TTTAGCAACTTGAGCGTAAATGGG3') and *cagAP2TA*(5'TTTAGCAACTTGAGTATAAATGGG3'), *cagAP3E* (5' ATCAATTGTAGCG TAAATGGG 3') and *cagA-PD* (5'TTGATTTGCCATCAA AATC 3') were used to amplify the EPIYA motif encoding the sequences A, B, C and D respectively.<sup>8</sup> Amplification of the *cagA3'* variable region was performed using primers *cag2*(5' GGAACCTAGTCGGTA ATG3') and *cag4* (5'-ATCTTTGAGCTTG TCTATC G 3').<sup>8</sup>

All PCR in this study were performed in a volume of 25µl containing 10XPCR buffer, 500nM of each primer, 2mmol/L MgCl<sub>2</sub>; 200µM each deoxy nucleotide triphosphate (dNTPs), 1.5U Taq DNA polymerase and 200ng DNA sample. PCR was performed in a thermal cycler (Applied Biosystem 2720) under the following conditions: initial denaturation at 94°C for five minutes was followed by 35 cycles of 93°C for 1 minute, 55°C for 30 seconds, 72°C for 1 minute and final extension at 72°C for 10 minutes. The amplified products were analyzed in 2% agarose gel by electrophoresis. Following electrophoresis, amplified product, the band of the sample was visualized under UV transilluminator. The bands were identified according to their molecular size by comparing with the molecular weight marker (100 bp DNA ladder) loaded in a separate lane.

**DNA sequencing:** To confirm the type and number of EPIYA motif, direct sequencing of *cagA* 3' variable region primer based amplified PCR product was performed. Sequencing was performed by Sanger dideoxy sequencing method at the Center for Advanced Research and Sciences, University of Dhaka.

For automated DNA sequencing, PCR products were purified by using Thermo Scientific GeneJet PCR Purification Kit prior to cycle sequencing. The purified DNA was quantified and purities were evaluated by spectrophotometer (Nanodrop, ND 1000, Japan).

After checking concentration of DNA, six samples were selected for sequencing. Purified products were sequenced using a BigDye Terminator Cycle Sequencing Kit in an ABI 3130 Genetic Analyzer (Applied Biosystems, USA). The sequences obtained were aligned using the CAP3 Sequence Assembly Program. After alignment, nucleotide sequences were transformed into amino acid sequences using the Blastx program and compared to previously published *CagA* gene sequence of strains *H. pylori* 26695 (AE000511) deposited into the GenBank.<sup>15</sup>

**Statistical Analysis:** After collection all data were checked, edited and analyzed by using computer based SPSS (Statistical package of social science) software. *p* value was calculated using Chi-square test to find the significant relationship. *p* value less than 0.05 was statistically significant.

## Results

Among 78 study population, 56 (71.8%) were male and 22(28.2%) were female with the mean age 39.38 ± 12.82 years (range 18-65 years). Of them majority (51, 65.4%) were diagnosed as gastritis followed by 11(14.1%) duodenal ulcer, 10(12.8%) gastric ulcer and 6(7.7%) gastric carcinoma. Out of 78 gastric biopsy specimens, 31(39.7%) were confirmed to have gastric *H. pylori* infection by rapid urease test (RUT) and *ureC* gene. Among 31 *H. pylori* positive cases, 19(61.3%) were *cagA* gene positive including 55%(11/20) of gastritis cases, 66.7%(2/3) of gastric ulcer cases, 66.7%(4/6) of duodenal ulcer cases and 100%(2/2) gastric carcinoma cases (table I). *cagA* gene is significantly associated with duodenal ulcer cases (*p*<0.05).

**Table I:** *H. pylori cagA* gene among *H. pylori* positive cases with different gastroduodenal diseases (n=31)

Diseases	<i>H. pylori</i> positive case	<i>H. pylori cagA</i> gene positive (%)
Gastritis	20	11(55)
Gastric ulcer	03	02(66.7)
Duodenal ulcer	06	04(66.7)
Gastric carcinoma	02	02(100)
<b>Total</b>	<b>31</b>	<b>19(61.3)</b>

Distribution of *cagA* EPIYA motif in *H. pylori cagA* positive cases and their relation to gastroduodenal diseases are shown in table II.

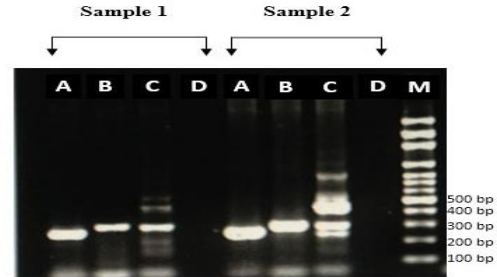
**Table II:** Distribution of *cagA* EPIYA motif in *H. pylori cagA* positive cases in relation to gastroduodenal diseases (n=19)

Diseases	CagA EPIYA motif (%)				
	AB	AC	ABC	ABCC	ABD
Gastritis(n=11)	01(9.1)	02(18.1)	06(54.5)	02(18.1)	00(0)
Gastric ulcer (n=02)	00(0)	00(0)	01(50)	01(50)	00(0)
Duodenal ulcer (n=04)	00(0)	00(0)	03(75)	01(25)	00(0)
Gastric carcinoma(n=02)	00(0)	00(0)	01(50)	01(50)	00(0)
<b>Total(n=19)</b>	<b>01(5.2)</b>	<b>02(10.5)</b>	<b>11(57.9)</b>	<b>05(26.3)</b>	<b>00(0)</b>

Among 19 *cagA* positive cases, EPIYA typing PCR showed that majority of gastroduodenal cases (57.9%) had 3 copies of EPIYA (ABC type), 26.3% had 4 copies (ABCC type), while remaining 10.5% had AC and 5.2% had AB type EPIYA motif. Most (75%) of the duodenal ulcer cases composed of ABC type EPIYA motif while 50% of gastric ulcer and gastric carcinoma cases had ABCC type EPIYA motif. There was no East Asian type *cagA* EPIYA ABD motif.

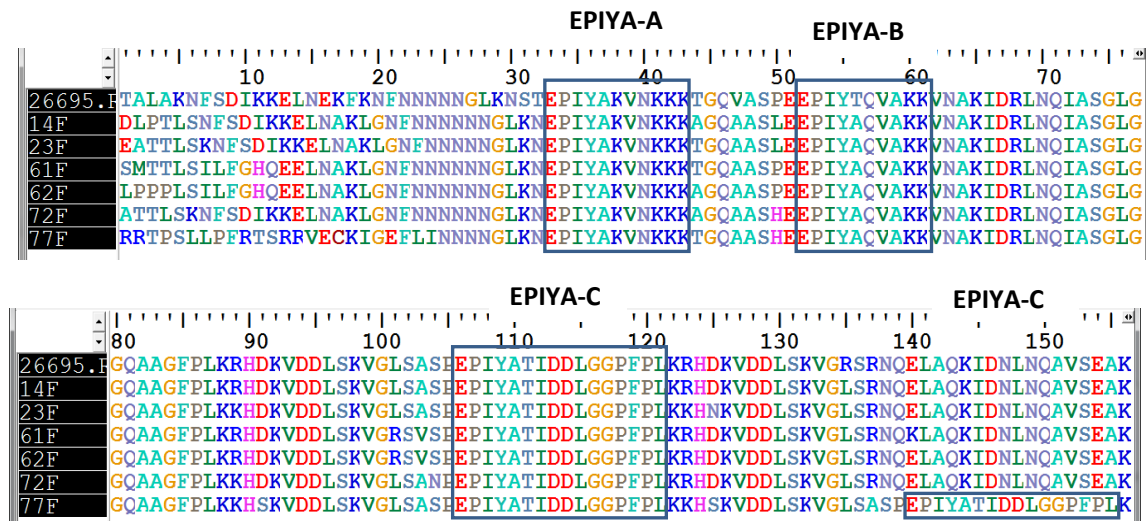
Nucleotide sequencing of the *cagA* 3' variable regions was performed for 6 selected cases including 2 from gastritis, 1 from gastric ulcer, 2 from duodenal ulcer and 1 from gastric carcinoma patients. Sequence analyses

confirmed that three types of EPIYA motifs were observed: EPIYA-A for EPIYAKVNKKK (A/T/V/S) GQ; EPIYA-B for EPIYA (A/T) (Q/K)VAKKVNAKI; and EPIYA-C for EPIYATIDDLGGPFPL (figure 2).



**Figure 1:** Amplification of *cagA* EPIYA motif by PCR from *H. pylori* strains. (1) ABC motifs and (2) ABCC motifs. M: 100-bp DNA marker.

No East Asian type of EPIYA-D (EPIYATIDFDEANQAG) was found. So the present study confirmed that PCR methods correctly classified the EPIYA motif types (i.e. strain-14, 23, 61, 62, 72 possessed ABC type, and strain-77 possessed ABCC type by PCR). The data are identical to data by sequencing.



**Figure 2:** Alignment of amino acid sequences among *cagA* strain (showing the EPIYA motifs) from 6 *H. pylori* strains including the *H. pylori* reference strain 26695.

## Discussion

*CagA* gene is considered to be a major virulence factor associated with gastric carcinoma. As large numbers of populations in Bangladesh are seropositive for *H. pylori* so, the present study

was done to detect the number and pattern of *H. pylori cagA* EPIYA motifs using PCR based typing and sequencing analysis to identify the *H. pylori* infected patients who are at risk to develop gastric carcinoma.

In this study, out of 31 *H. pylori* positive cases, 19(61.3%) were *cagA* gene positive. This finding is consistent with the findings of previous studies showing *cagA* positivity rate 61% in china, 65.9% in Brazil and 68.4% in Bangladesh.<sup>12,15,16</sup>

In the present study *cagA* gene was positive in 66.7% cases in each of duodenal ulcer and gastric ulcer patients compared to 55% cases of gastritis. *CagA* gene was significantly associated with duodenal ulcer cases ( $p < 0.05$ ). This finding is in agreement with studies done in Japan and Cuba but in contrast to a study done in Singapore reported that there was no association between *cagA* status and duodenal ulcer.<sup>17-19</sup> For this difference in the *cagA* status, one possibility is the large genomic variations in the *H. pylori* genomes that amplifies the *cagA* gene from *H. pylori* isolated in one country failed to detect *cagA* in isolates from another country.

Western type *cagA* EPIYA-C motif was found in all 19 *cagA* positive *H. pylori* cases but there was no East Asian *cagA* EPIYA-D motif. Similar findings were reported from India and Colombia.<sup>11,20</sup> In contrast, these results differed from that of Korea where the East Asian type *cagA* (EPIYA-D) is dominant and some other countries like Malaysia and Thailand where both types of *cagA* (Western and East Asian) are present.<sup>21-23</sup>

Among 19 *cagA* positive cases majority (11, 57.9%) were EPIYA ABC motif followed by 5(26.3%) EPIYA ABCC, 2(10.5%) AC and 1(5.2%) AB motif. These results are comparable with the results of previous study done in Colombia.<sup>24</sup> The multiple numbers of EPIYA motifs, especially EPIYA-C, is thought to be related to the development of gastroduodenal diseases. *CagA* positive *H. pylori* strains with multiple EPIYA-C motifs are reported to be associated with a higher gastric cancer risk than strains with only one EPIYA-C motif.<sup>8,25</sup> In the present study more than one EPIYA-C (ABCC) was found in 26.3% of *cagA* positive cases which is comparable to the findings reported in South Africa and Colombia.<sup>24,26</sup> Geographic variations in the frequency of *H. pylori* strains with one or two EPIYA-C repeats may be explained by different acidic conditions in the gastric mucosa, which could be related to differences among populations in the frequencies of cytokine gene

polymorphisms that attenuate gastric acid secretions.<sup>27</sup>

Present study showed that the variation in the EPIYA motifs in *CagA* protein was not directly associated with the outcome of the disease caused by *H. pylori* and that there is no relation between the number of EPIYA-C motifs and the gastroduodenal diseases ( $p > 0.05$ ). Higher number of EPIYA C segments was associated with gastric carcinoma in Italy and Brazil, otherwise no association was observed in Colombia.<sup>24-25</sup> These differences might be due to different study designs, sample size, populations and geographical diversity of *H. pylori* markers of pathogenicity.

## Conclusion

This study shows the pattern of *cagA* EPIYA motif in gastric biopsy specimen from patients with gastroduodenal diseases. Possibly this is the first study in Bangladesh that shows Western type *cagA* EPIYA motif was found in all 19 *cagA* positive *H. pylori* cases and there is no East Asian EPIYA motif. This finding demand further study to be conducted with more clinical specimens and phylogenetic analysis of the *H. pylori* genome to determine the ancestry relationship among the overall international isolates. The multiple numbers of EPIYA motifs, especially EPIYA-C, is thought to be related to the development of severe gastroduodenal diseases and associated with higher gastric cancer risk and was found in 26.3% of *cagA* positive cases in the present study which also demand more studies to be conducted further to estimate the actual risk of severe gastroduodenal diseases.

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