

Prevalence and Diversity of *Cag* (PAI) in *Helicobacter pylori*: Study on Gastric Ulcer

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Abstract

Background: Frequency of *H. Pylori* in northern part of Iran within normal population is about 90% which is high, particular marker in order to diagnose patients with higher risk of peptic ulcer disease progression would be helpful for clinical practice. The purpose of this study was to evaluate the variable locus of *CagA* gene in virulence form.

Methods: For this aim 200 types of biopsy infected and positive urease test and 20 samples of healthy people biopsies in order for control were provided. After DNA purification, the variety of locus in *CagA* gene was confirmed by *glmM*, *SSA* and *CagV* primers.

Results: The results showed that 142 typical were positive for *CagA*. *CagV* primer used to variety fixed. The *CagV* gene exposed a special genetic variety in the 800 bp, 825 bp, 850 bp, 900 bp areas.

Conclusions: The result of study on this locus suggested that variation of *CagV* could be consider as a paradigm in intensity and distribution of the gastric ulcer disease and key marker to prevent progression or inhibition of the progress in gastrointestinal disease.

Keyword:

CagA (PAI), *CagV*, *H. Pylori*, PCR

Introduction

H. pylori colonizes in the mucosa of the human stomach where it establishes a long-term infection associated with acute or chronic gastric inflammation, which may progress to peptic ulcer disease, atrophic gastritis with intestinal metaplasia, or gastric cancer. A variety of clinical outcomes of *H. pylori* infection are associated both with host factors and with bacterial virulence factors [1]. Several *H. pylori* virulence genes have been identified, of these, *oipA*, *vacA*, *CagA* and *babA* appear to play a major role in pathogenicity. The cytotoxin-associated gene (*CagA*) is a marker for the *cag* pathogenicity island (PAI), a 40-kb genomic region [1,2]. Most strains of *H. pylori* from patients with peptic ulcer disease carry the *CagA* gene (*CagA* positive strains) and the presence of the *CagA* gene increases the risk of developing peptic ulceration, atrophic gastritis, and adenocarcinoma in the stomach [1]. Consequently the discrimination of *CagA* positive and *CagA* negative *H. pylori* strains might prove useful in predicting the chance for complications as well as for clinical and epidemiological studies of *H. pylori* infection [1,2].

H. pylori *cag* PAI status is typically assessed by the immune response to the immuno-dominant *CagA* or by detection of the *CagA* gene. Available serological tools to characterize the infecting *H. pylori* strains have been questioned because of their inadequate sensitivity and specificity [3]. Mutational analysis in *H. pylori* showed the importance of *CagV* presence for *CagA* translocation in host cells so that *CagA* could cross the membrane barrier when *CagV* function has been completed [4,5]. As the frequency of *H. pylori* in northern part of

Iran within normal population is about 90% which is high, particular marker in order to diagnose patients with higher risk of peptic ulcer disease progression would be helpful for clinical practice, as specific as *H. pylori* genotyping from isolate with positive urease tests in the laboratory. In present study, the frequency of cytotoxin-associated gene A (*CagA*) was determined and the variant locus of *H. pylori* defined using three specific primers of *glmM*, *SSA* and *CagV*.

Materials and Methods

Patients

At first, patients suspicious to gastric ulcer disease and *Helicobacter pylori* infection which refer to the physician were determined. After performing gastric endoscopy and biopsy by physician which obtained from antrum and corpus at stomach, the presence of *H. pylori* from obtained biopsies was checked by culturing on urease media in the first phase. 200 patients with positive test result were selected for further study. Afterward, the biopsies transferred to the laboratory for tracing of *H. pylori* infection and pathogenesis by performing PCR; also 20 patients with negative test result were selected as case-control study. This study was approved by the local ethics committee and patients gave written informed consent.

Culture

Antral biopsy specimens were used for bacterial culture. Briefly, biopsies were collected in one ml sterile thioglycolate broth. Immediately after biopsy collection, samples were homogenized in the sample medium with a sterile syringe. The homogenate dilution was subsequently plated on Columbia agar (CA) plates containing 7%

defibrinated sheep blood, 7% fetal calf serum and antibiotics (vancomycin 2.0 mg, polymyxin 0.05 mg, trimethoprim 1.0 mg). The CA plates were incubated for 7 days under microaerophilic conditions (5% O₂, 10% CO₂ and 85% N₂; Binder-USA) at 37°C and in a water-saturated atmosphere. Suspected colonies were identified as *H. pylori* based on their colony morphology, their shape as determined by Gram staining and positive biochemical tests for catalase, oxidase and urease activity. A single colony of each patient was randomly selected from the primary culture plate, then multiplied on CA plates and stored at -20°C in 20% glycerol for future testing [6].

Total DNA extraction

Bacteria from the -20°C stocks were freshly grown on CA medium plates and chromosomal DNA was extracted using a commercially available DNA isolation kit (Roche).

H. pylori genotyping

After DNA extraction, polymerase chain reactions (PCR) were performed in a volume of 50 µL. The PCR primers are listed in Table 1. A set of primers (P1 and P2) that amplified SSA and *glmM* used for detection of *H. pylori* three locus and for variation determining a set of *CagV* (F, R) used moreover [7]. In order to keep track of *CagA* gene, *CagA* primers were used also. Amplification was performed under the following conditions for SSA: initial denaturation at 98°C for 10 min, followed by 37 cycles of denaturation at 92°C for 30 s, annealing at 64°C for 60 s and extension at 72°C for 2 min. PCRs of *glm*, initial denaturation at 93°C for 1 min, followed by 35 cycles of denaturation at 93°C for 60 s, annealing at 55°C for 60 s and extension at 72°C for 60 s. PCRs of *CagA*, initial denaturation at 95°C for 60 s, followed by 35 cycles of denaturation at 95°C for 60 s, annealing at 55°C for 60 s and extension at 72°C for 60 s. PCRs of *CagV*, initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 60 s, annealing at 51°C for 60 s and extension at 72°C for 60 s. Analysis of PCR products was performed by electrophoresis on 6% polyacrylamide gel, followed by staining with ethidium bromide.

Primer	Product size (bp)	Primer sequence (5'→3')
SSA	303 bp, 474-776	5'-TGCGTGTCTATTGACAGCGAGC-3'
		5'-CCTGCTGGGCATACCTCCAG-3'
<i>glm</i>	294 bp, 784-1077	5'-AAGCTTTTAGGGGTGTTAGGGGT-3'
		5'-AAGCTTACTTTCTAACACTAACGC-3'
<i>CagA</i>	349 bp, 1228-1576	5'-GATAACAGGCAAGCTTTTGAGG-3'
		5'-CTGCAAAAGATTGTTGGCAGA-3'
<i>CagV</i>	800 bp	5'-CAAAGATAACGGATAAAGT-3'
		5'-CTGTTAGTAGCGTAATTGTC-3'

Table 1: Primer sequences for human HP SSA, *glmM*, *CagA* and *CagV*.

Statistical analysis

Statistical analysis was performed using χ^2 exact tests, using Minitab 15 with confidence interval of 95 percent which consider *H. pylori* strain was either independent of the *CagA* presence/absence and the clinical outcome.

Results

From all 220 prepared isolate, seven control were women (35%) and thirteen (65%) were men, the sex ratio being 1:1.8, with a mean age of 38.7 years. In the peptic ulcer group, 80 were woman (40%) and 120 were men (60%), the sex ratio being 1:1.5, with a mean age of 52 years. Since the p value of the positive PCR, sexes and age group is greater and equal than 0.05 respectively so the correlation is not significant.

Amplification of genes

After running the samples in a thermo-cycler, PCR products were electrophoresed on 6% polyacrylamide gels, stained with silver nitrate and following results obtained.

SSA locus

PCRs yielded satisfactory products with expected size and reproducible results (Figure 1). Negative PCRs were repeated for confirmation, along with positive controls. The PCR product size of the SSA varied in length from 474 to 776 nt. PCR primer pairs derived from the *CagA* can be seen in the area of 303 bp. From total 200 samples, 156 (78%) were positive for this pair of primers (Table 2).

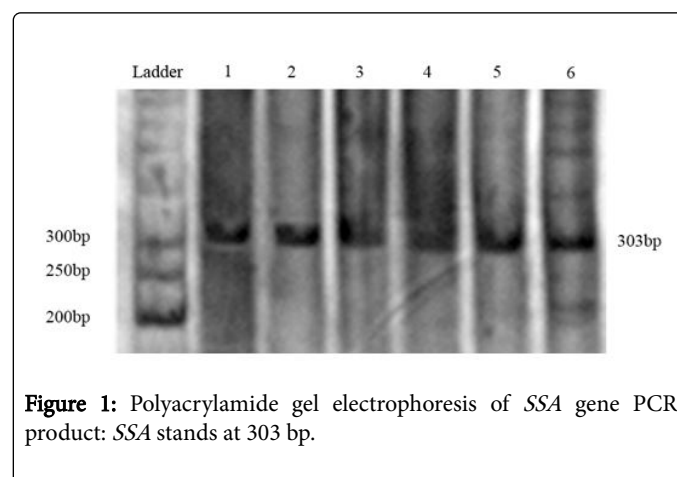


Figure 1: Polyacrylamide gel electrophoresis of SSA gene PCR product: SSA stands at 303 bp.

Primer	Allele based on bp	Positive	Negative	Total
SSA	303	156	44	200
<i>glmM</i>	297	168	32	200
<i>CagA</i>	349	142	14	156
<i>CagV</i>	I=800	I=34		126
	II=825	II=32		
	III=850	III=30		
	IV=900	IV=30	-	

Table 2: Distribution of *H. pylori* SSA, *glmM*, *CagA* and *CagV* genes in the study groups using PCR method.

glmM locus

PCR conditions for *glmM* were followed without initial denaturing step of DNA. PCRs yielded satisfactory products with expected size and reproducible results (Figure 2). Negative PCRs were

repeated for confirmation, along with positive controls. The PCR product size of the *glmM* varied in length from 784 to 1079 nt. PCR primer pairs derived from the *CagA* can be seen in the area of 296 bp. Line No. 1 present the negative control (not ulcerative). After running 200 samples on the gel, 84% (168 of 200 samples) were positive for this pair of primers (Table 2).

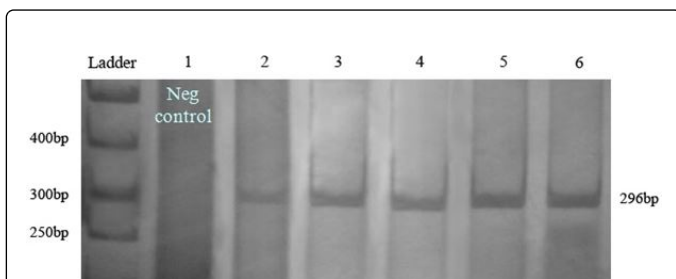


Figure 2: Polyacrylamide gel electrophoresis of *glmM* gene PCR product: *glmM* stands at 296 bp.

CagA locus

PCR conditions for *CagA* were followed as status of the *glmM* program without initial denaturing step of DNA. From 156 samples which were positive for both of *glmM* and *SSA*, 142 samples (91%) were positive for *CagA* gene. The PCR product size of the *CagA* varied in length from 1228 to 1576 nt. PCR primer pairs derived from the *CagA* can be seen in the area of 349 bp. PCRs yielded satisfactory products with expected size and reproducible results (Figure 3). Negative PCRs were repeated for confirmation, along with positive controls.

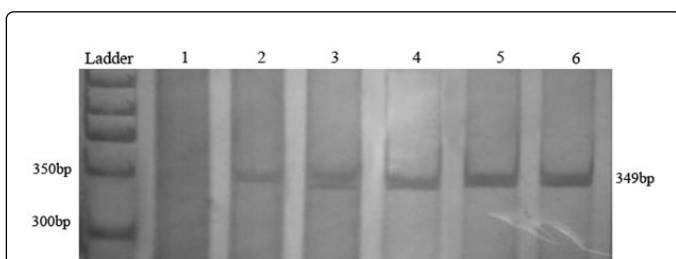


Figure 3: Polyacrylamide gel electrophoresis of *CagA* gene PCR product: *CagA* stands at 349 bp.

CagV locus

This pair of primer used for detecting variable sequence. After running PCR products on polyacrylamide gels, different sizes exposed. The size of 800 bp bands optimized for a number of samples and for others 825 bp, 850 bp and 900 bp observed and categorized to sub genotypes I, II, III, IV (Figure 4).

After considering 142 samples that were positive for *CagA* primers, 126 cases (88.7%) was positive for *CagV* that 34 samples of the 126 positive samples (27%) in group I, 32 samples (25.3%) in group II, 30 (23.9%) in group III, and finally 30 (23.9%) were in group IV.

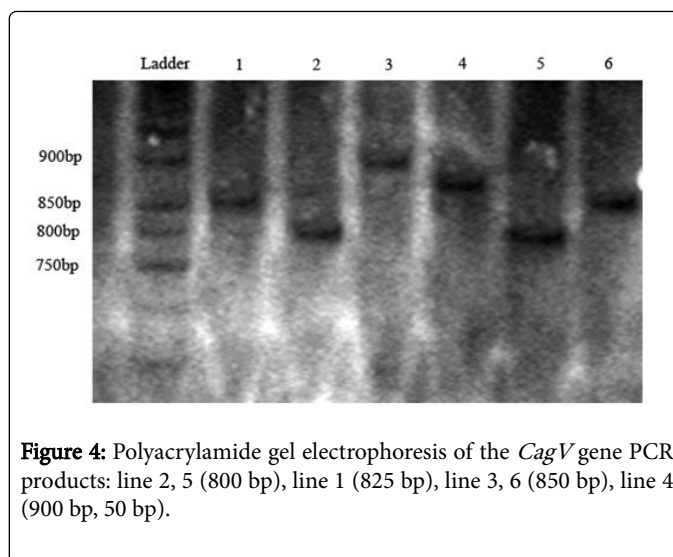


Figure 4: Polyacrylamide gel electrophoresis of the *CagV* gene PCR products: line 2, 5 (800 bp), line 1 (825 bp), line 3, 6 (850 bp), line 4 (900 bp, 50 bp).

Statistical analysis

There was no significant relationship between *CagA* gene presence and gastric ulcer in clinical samples.

Discussion and Conclusion

Helicobacter pylori is a spiral bacterium that inhabits the gastric mucosa of the human stomach in approximately half of the world's population for a life time [8]. Infection of this unique ecological niche by *H. pylori* induces gastric mucosal inflammation, which may progress into peptic ulcer [9].

Persistent infection also increases an individual's risk for development of gastric adenocarcinoma and gastric MALToma [10]. Recent studies have shown that different *CagA* and *iceA* *H. pylori* genotypes can lead to different clinical outcome consequences in certain populations [11,12]. *CagA* is the virulence factors of *H. pylori* which its function is under consideration. *CagA* gene contains repetitive sequences in the region of 3' end [13-16].

In research which done by Yamaoka et al. and followed by Pan et al. they found that the largest chain of *H. pylori* in China which cause to gastric ulcers were *CagA* positive [8,14]. In another study by Zhou et al for detection of *H. pylori* using PCR, in first study 18 samples that were positive for *H. pylori*, 17 of them were positive in *CagA*. In the second study they prepare 82 samples which 79 samples were positive for *CagA* gene also [17-19].

According to other research and our result, the relation between *CagA* gene and infection of *H. Pylori* will be confirmed. The results show that in most places *CagA* gene activated in *H. pylori* that can be infection is more than 90%. The used sequence primers for *SSA* locus in this study were same as that Smith et al used in Africa and Zhou et al. in China [13,20]. Obtained result of this study and other research results suggested that this gene is a well candidate for specific detection of *H. pylori* [17,21].

According to the study which have done by Smith et al used in Africa, Zhu et al. in China and specially van Doorn et al. that after analyzing on 176 sample which were negative on culturing and even CLO test, after PCR reported positive; The bacteria detection by specific primers and performing PCR have more accurate answer we

give [13,14,20,22]. The study was conducted that the detection of *H. pylori* strains with urease genes (*glm*) is more accurate.

The primers that used for determination of variable 3' area in this study were the same as used in [18]. Zhou et al. in their study exposed that *CagA* positive samples (71 from 77 samples) were positive for *CagV* and were also different in terms of the molecular weight band received in regions of 825 bp to 950 bp. In another study, Yamaoka et al. observed these 4 bands in the regions of 750 bp to 900 bp [18,19].

This variability in the rates among different studies including this study may be attributable to the differences in the methods of identification in different studies, different demographic distribution of the bacteria among various regions, and previous antibiotic consumption [21,23]. These reasons may also explain the different distribution of this organism among different international studies [17,24]. Almost the results suggested this locus as a key marker in order to prevent progression or inhibition of the progress in gastrointestinal disease as well as a paradigm in intensity and distribution of the gastric ulcer disease. The result of study on this locus at this moment reveals that variation of *CagV* is similar to east of Asian but it need more consideration on motifs number of EPYIA in Iran [25].

Authors Declaration

The authors declare that they have no conflict of interest.

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