Anti-CagA IgG Antibody Is Independent from *Helicobacter pylori VacA* and *CagA* Genotypes

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ABSTRACT

Background: *Helicobacter pylori* strains have two classical virulence genes, the cytotoxin-associated A (*cagA*) gene and the vacuolating cytotoxin A (*vacA*) gene, which are located in the *cag* pathogenicity island (*cag*PAI). Serum immunoglobulin G (IgG) antibodies to *H. pylori*, especially, the CagA antigen may be a reliable marker for selection of dyspeptic patients for upper endoscopy.

***Methods*:** Serum sample of 129 dyspeptic patients with positive *H. pylori*, were tested for serum IgG Anti-CagA antibody by ELISA. The presence of the *cagA* and *vacA* genotypes were determined using polymerase chain reaction (PCR) on biopsy samples taken via endoscopy.

Results: Positive serum IgG anti-CagA antibodies in patients with $cagA^{+}/vacA^{+}$ and $cagA^{+}/vacA^{-}$genotypes were 22/23 (95.6%) and 18/19 (94.7%), respectively. In addition, serum IgG anti-CagA antibodies in patients with $cagA^{-}/vacA^{+}$ and $cagA^{-}/vacA^{-}$ genotypes were 22/47 (46.8%) and 33/40 (82.5%), respectively.

Conclusion: It can be concluded that the serum IgG anti-CagA antibody alone could select patients with dyspepsia following upper endoscopy. The assessment of vacuolating cytotoxin activity of *H. Pylori* is, therefore, not required, even when *vacA* gene is positive. This hypothesis needs to be studied in a large number of patients with dyspepsia.

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Introduction

Helicobacter pylori is a gram-negative, microaerophilic bacterium that colonizes more than half of the world's human population. Despite the development of gastric mucosal inflammatory response and *H. pylori*-specific humoral immune response, *H. pylori* can persistently colonize the stomach for decades or for life. Most individuals harboring *H. pylori* remain asymptomatic, but the presence of this organism is a risk factor for the development of peptic ulceration, gastric mucosa-associated lymphoid tissue (MALT) lymphoma, and gastric adenocarcinoma (1-3).

The *cag* pathogenicity island (*cag*PAI) is the most virulence factors of *H. pylori* which are frequently associated with the most serious clinical outcomes. The *cag*PAI can be divided into two parts: the upstream *cagII* and the downstream *cagI* regions by an insertion sequence called IS605. The *cagA* gene is located in the most downstream portion of *cagI* and is known as a marker for the *cagI* region. The *cagA*-positive strains are reported to be related to severe clinical outcomes, especially in Western countries (4). Two virulence factors have been found more frequently in *H. pylori* strains isolated from patients with ulcers or cancer than in strains isolated from patients with gastritis. The former is the cytotoxin-associated antigen (CagA) which reflects the presence of the *cag* PAI, including about 30 genes of unknown function (5, 6). Expression of CagA protein is closely associated with that of vacuolating cytotoxin, although the underlying mechanism is not understood. Another important virulence factor of *H. pylori* is a vacuolating cytotoxin activity (VacA), which is associated with injury of epithelial cells. The *vacA* gene present in nearly all strains of *H. pylori*, but it is polymorphic, comprising variable signal regions (type *s1*or *s2*), mid-regions (type *m1*or *m2*) and recently *i* region (7, 8). Furthermore, vacuolating cytotoxin activity expression blocks T-cell activation, proliferation and inhibits antigen presentation in T cells. In addition, antigen processing by B lymphocytes is blocked, possibly by limiting the maturation of endosomes to MHC class II compartments where antigen loading takes place. Therefore, vacuolating cytotoxin activity limits T and B-cell response against *H. pylori* (9-11).

Chronic *H. pylori* infection elicits local and systemic immunologic response leading to production of IgG antibody. IgG antibody can be detect against whole-cell preparation in serum and also against cytotoxin-associated protein. This protein is highly immunogenic and usually stimulates immune response and can be measured by ELISA and western bloating system (12)

It has been shown that a specific antibody response pattern is one of the most reliable methods for diagnosis of *H. pylori* infection especially peptic ulcer disease. The aim of this study was to extend the data and to search for specific antibody patterns in sera from *H. pylori* patients having *cagA* gene with or without *vacA* gene, in patient with dyspepsia.

Materials and Methods

Patient Samples

The present study carried out in 130 patients with the chief complaint of dyspepsia with over 2 month duration who were referred to the endoscopy ward of Firoozgar Hospital. The patients, who were aged between 16 and 69 years, were selected according to Leeds Medical School criteria (13). All the patients provided informed consent and accepted to complete a standard questionnaire. Esophago-gastroduodenoscopy (EGD) was done for all the patients in the same center by expert endoscopists. The patients with dyspepsia with or without peptic ulcers were selected and included in the study. The eligibility of the patients was based on the results of the questionnaire and EGD. Those patients who had no history of proved ulcer such as previous *H. pylori* eradication, cigarette smoking ,malignancy and other underlying diseases in EGD, and did not use antibiotics (at least 2 months before endoscopy) were excluded from the study. Four biopsy specimens were taken from the antrum and

gastric body for histological study and *H. pylori* detection. All the formalin embedded specimens were fixed and stained with Hematoxylin and Eosin Stain (H&E) or Geimsa. The specimens were evaluated by an experienced pathologist. A patient was considered to be positive for *H. Pylori* when at least 5 bacilli in each microscopic field were found. Five ml of the blood sample was taken and sent to the Immunology laboratory for anti-CagA antibody measurement. Antibody measurement was performed using the ELISA commercially supplied (Diagnostic Bioprobes- Italy). The anti-CagA antibody in the patients' sera was based on the synthetic CagA coated microplates. The complex was detected by HRP conjugated antisera, and the color that developed was measured in 450-nm filters prefiltered at 620 nm. The optical density (OD) of each well was measured and the antibody level was calculated using a standard calibration curve. The method is both sensitive and specific (98%), and its diagnostic sensitivity and specificity are more than 98%. A value ≥ 5 Arbitrary/ml was considered as positive for the anti-CagA antibody.

PCR

The genomic DNA was extracted from the biopsy samples using a DNA isolation kit for cells and tissues (Roche Applied Science Company, Germany) in according to the manufacturer's instruction and stored at -20 °C. Two sets of primers were designed complementary to the sequence located within the conserved region of the gene primers; *cagA*1 and *cagA*2. The primers sequences and size are depicted in Table 1 (14).The amplified products were detected after agarose gel electrophoresis with a 340 bp which indicated the presence of the *H. pylori-cagA* in the specimen (Figure. 1). The identification gene product belong to *H. pylori* was confirmed via the PCR for the *GlmM* (urea C), a conserved gene, specific for the bacteria (14). The identification of *vacA* gene was confirmed by PCR using primers specific for its signal sequence with the pair of primers; *vacA*1 and *vacA*2. The characteristic of the primers is illustrated in table1. The amplified product was evident as above with 480 bp band which indicates the presence of the *H. pylori vacA* in the specimen (Figure. 2).

Table 1. Characteristics of the primers used for the detection of *cagA* and *vacA genes*.

Amplified region	Primer designation	Primer sequence	Product size (bp)	Ref.
GlmM	GlmM-F GlmM-R	5'-AA GCT TTT AGGGG TGTTAGGG GTTT-3' 5'-AA GCT TAC TTT CTAA CACTAACGC-3'	294	14
cagA1	CAG1-F CAG1-R	5'-GAT AAC AGG CAA GCT TTT GAG G-3' 5'-CTG CAA AAG ATT GTT TGG CAG A-3'	349	14
cagA2	CAG2-F CAG2-R	5'-TTG ACC AAC AAC CAC AAA CCG AAG-3' 5'-CTT CCC TTA ATT GCG AGA TTC C-3'	1385	14
vacA1	VAC1-F VAC1-R	5'-CTG CTT GAA TGC GCC AAA C-3' 5-CAC AGC CAC TTT CAA TAA CGA-3'	71	14
vacA2	VAC2-F VAC2-R	5'-ATG GAA ATA CAA CAA ACA CAC-3' 5'-CGT CAA AAT AAT TCC AAG GG-3'	480	14

Statistical Analysis

All the data were analyzed using SPSS software (version18) for each patient. Age is shown with ± standard deviation. The effects of *vacA* gene positive on the serological response were expressed as odds ratios (ORs) with 95% confidence intervals (CIs) with reference of *vacA* gene negative patients having *H. pylori* infections. The results were compared by Mantel-Haenszel chi-squared test with Yates correction or Fisher exact probability test. A *P*-value less than 0.05 was considered statistically significant.

Results

Overall, 130 patients, with ulcer and non-ulcer dyspepsia were selected based on the results of a questionnaire and esophagogastroduodeno-scopy. One of these patients had *vacA* gene positive and negative result which was excluded. The remaining of 129 patients, 42/129 (32.5%) and 87/129 (67.5%) had *cagA* gene positive and negative, respectively. The average age was 42.9 ±12.1 (16 to 64) years in the *cagA* gene positive

patients and 40.9±14.7 (16-64) years in patients with *cagA* gene negative. Amongst the patients, 20/42 (47.6%) and 45/87 (51.7%) of patients were female in *cagA* gene positive and negative patients, respectively. Also, 23/129 (17.8%) and 19/129 (14%) of patients had $cagA^{+}/vacA^{+}$ and $cagA^{+}/vacA^{-}$, respectively. In addition, 12/23 (52%) of patients with $cagA^{+}/vacA^{+}$ and 7/19 (36.8%) of patients with $cagA^{+}/vacA^{-}$ were females. The mean age ±SD of patients with $cagA^{+}/vacA^{+}$ was 39.0±13.6 (16-67) years and the mean age ±SD of patients with $cagA^{+}/vacA^{-}$ was 40.0±14.7 (18-69) years.

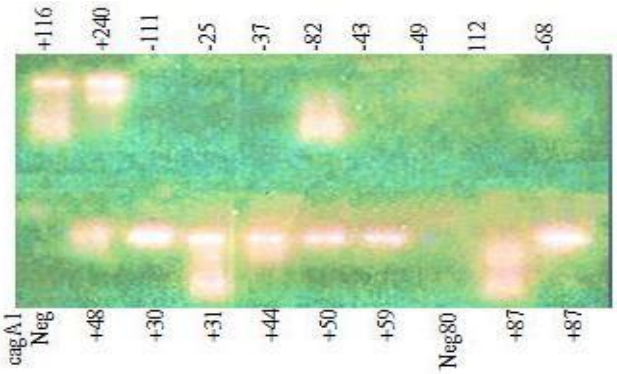

Figure 1. $cagA^{+}$ and $cagA^{-}$ samples. Numbers indicate the code of each patient.

The patients with *cagA* gene positive group, 23/42 (54.8%) and 19/42(45.2%) had *vacA* gene positive and negative, respectively. The patients with *cagA* gene negative group, 47/87 (54%) and 40/87 (46%) had *vacA* gene positive and negative, respectively. Serum IgG anti-CagA antibodies positive in patient with $cagA^{+}/vacA^{+}$ and $cagA^{+}/vacA^{-}$ genotypes were 22/23 (95.6%) and 18/19 (94.7%), respectively. Serum IgG anti-CagA antibodies in patients with $cagA^{-}/vacA^{+}$ and $cagA^{-}/vacA^{-}$ genotypes were 22/47 (46.8%) and 33/40 (82.5%), respectively. These results are presented in table 2. The relation of serum IgG anti-CagA antibodies between two groups, $cagA^{+}/vacA^{+}$ and $cagA^{+}/vacA^{-}$ were not significant (odds ratio: 1.2, 95% CI: 0.8-16.6; p=0.9) (Table 3).

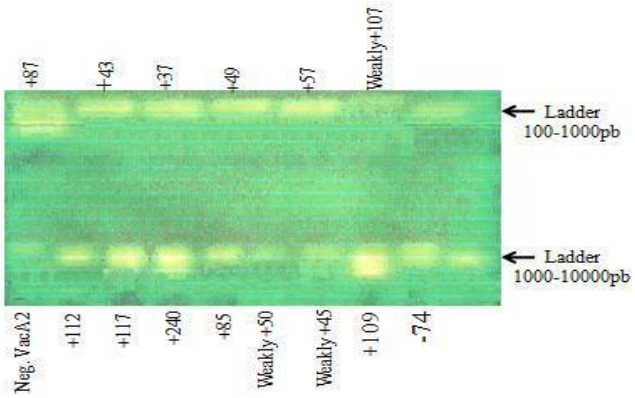

Figure 2. $cagA^{+}$ and $cagA^{-}$ samples. Numbers indicate the code of each patient.

Table 2. *H. pylori vacA* and *cagA* genotypes and IgG anti CagA antibodies in 129 patients. .

Genotypes		n(%)	IgG anti *Cag A* antibody n(%)
VacA	*CagA*		
+	+	23/129(17.7)	22/23(95.6)
-	+	19/129(14.6)	18/19(94.7)
+	-	47129(36.2)	22/47(46.8)
-	-	40/129(30.8)	33/40(82.5)

Table 3. IgG anti CagA antibody in patients with *vacA* gene positive and negative in patients with *cagA* gene positive group.

IgG anti Cag A antibody	Number of *vacA* gene		Odds Ratio (95% CI)	P-Value
	Positive(%)	Negative(%)		
Positive	22(95.6)	18(94.7)	1.2 (0.8-16.6)	0.9
Negative	1(4.5)	1(5.3)		

Discussion

Our results did not show the relation of IgG anti-CagA antibodies in patients with *cagA*$^{+}$/*vacA*$^{+}$ genotypes (22/23, 95.6%) and *cagA*$^{+}$/*vacA*$^{-}$ (18/19, 94.7%) genotypes in patient with dyspepsia (odds ratio: 1.2, 95%CI: 0.8-16.6; p=0.9). The serological response to *H. pylori* antigens were heterogeneous. Although, the serum IgG anti-CagA antibody results might be different in another study with *H. pylori-vacA* alleles, suggesting that some patients with *vacA* gene positive do not have vacuolating cytotoxin activity.

The *vacA* gene of *H. pylori* encodes for VacA, a secreted vacuolating cytotoxin,which induces a vacuolating cytotoxic effect in gastric cell lines. The *vacA* is a polymorphic gene and both active and inactive forms of the toxin exist. Strains of *H. pylori* that express active forms of the toxin are associated with more severe cases of the disease. Three regions of variation have been defined and there are at least two primary variants in each region; the regions are designated as the signal (*s*), intermediate (*i*), and middle (*m*) regions. Furthermore, strains carrying *vacA s1*, *i1*, *m1*, and combinations of these alleles are overall associated with more severe diseases. Each region was found in different location of the genome and have distinct function (8). A large variety of additional cytotoxic functions has been attributed to VacA in the last 10 years of extensive characterization, such as altering the endosomal function, inhibiting T-cell proliferation, internalizing and damaging mitochondria, and inducing apoptosis (15).

The *cag*PAI is a pathogenicity island approximately 40 kb in size containing about 30genes including those which encode the type IV secretion system (TFSS), a syringe-like structure responsible for transfer of the CagA protein from *H. pylori* into the host cell (5, 6). Xiang *et al* classified *H. pylori* strains into two groups, type I and type II. Type I strains is positive for both *cagA* gene and vacuolating cytotoxin activity and type II strains positive for *cagA* gene without vacuolating cytotoxin activity, although *vacA* gene is present. The gene coding for the vaculating cytotoxin, VacA, is polymorphic and present in all strains, but various strains have shown marked differences in the production of vaculating cytotoxins. Type I was strongly associated with peptic ulcer diseases in the host (7). When the *cagA* positive gene are presented, immune system usually have been exposed to the CagA antigen, especially since CagA is injected into epithelial cell of stomach by TFSS which is encoded by other genes contained within the PAI. The vacuolating cytotoxin activity expression blocks T-cell activation, proliferation and inhibits antigen presentation in T cells. In addition, antigen processing by B lymphocytes is blocked, possibly by limiting the maturation of endosomes to MHC class II compartments where antigen loading takes place. Therefore, vacuolating cytotoxin activity limits the T and B-cell response against *H. pylori* (9-11).

It was reported, the concomitant presence of *cagA*$^{+}$ and *cagA*$^{-}$ *H. pylori* organisms in the same patients, and also cytotoxic and non-cytotoxic *H. pylori* strains were reported at the same time in the same biopsy samples. Our data also suggest that mixed infection with *vacA*$^{+}$ and *vacA*$^{-}$ *H. pylori* strains is found in Iranian patients which could allow for this bacteria to establish a persistent infection (16).

The epidemiology of *H. pylori* has been extensively studied during the past two decades. The majority of investigations have utilized endoscopic diagnosis, CLO-test, breath test, serology, and molecular survey of the *H. pylori* bacteria to delineate the natural history and clinical epidemiology of the associated diagnoses (17). Therefore, serum IgG antibodies to *H. pylori*, especially, the CagA antigen may be a reliable marker for selection of dyspeptic patients for upper endoscopy.

To our knowledge this is the first study in Iranian patients. The study could have resulted differently it was solely based on *vacA* alleles. The measurement of serum IgG anti VacA

antibody, and small number of patients are limitations of this study.

In conclusion, the serological response to *H. pylori* is heterogeneous, but serum IgG anti-CagA antibodies alone could select patients with dyspepsia for upper endoscopy, therefore assessment of vacuolating cytotoxin activity of *H. pylori* was not required, even though *vacA* gene was positive. This hypothesis is required to study in a large number of patients with dyspepsia.

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Conflict of Interest

The authors declare no conflicts of interest.

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