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Association of *Helicobacter pylori cagA* Gene with Gastric Cancer and Peptic Ulcer in Saudi Patients

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This study was conducted to assess the relationship between occurrence of gastric cancer and peptic ulcer, and the presence of *H. pylori cagA* gene and anti-CagA IgG, and to estimate the value of these antibodies in detecting infection by *cagA* gene-positive *H. pylori* strains in Saudi patients. The study included 180 patients who were subjected to upper gastrointestinal endoscopy in Taif province and Western region of Saudi Arabia (60 gastric cancer, 60 peptic ulcer, and 60 with non-ulcer dyspepsia). Gastric biopsy specimens were obtained and tested for *H. pylori* infection by rapid urease test and culture. PCR was performed on the isolated strains and biopsy specimens for detection of the *cagA* gene. Blood samples were collected and tested for CagA IgG by ELISA. *H. pylori* infection was detected among 72.8% of patients. The *cagA* gene and anti-CagA IgG were found in 63.4% and 61.8% of *H. pylori*-infected patients, respectively. They were significantly ($p < 0.01$) higher in patients with gastric cancer and peptic ulcer compared with those with non-ulcer dyspepsia. Detection of the CagA IgG was 91.6% sensitive, 89.6% specific, and 90.8% accurate compared with detection of the *cagA* gene. Its positive and negative predictive values were 93.8% and 86%, respectively. The study showed a significant association between the presence of the *cagA* gene and gastric cancer and peptic ulcer disease, and between anti-CagA IgG and the *cagA* gene in Saudi patients. However, a further larger study is required to confirm this finding.

Keywords: *Helicobacter pylori*, gastric cancer, peptic ulcer, *cagA* gene, anti-CagA IgG

Introduction

Helicobacter pylori (*H. pylori*) is a clinically important pathogen that colonizes about 50% of the world's population [4]. Although infection is found worldwide, higher rates have been reported in developing countries compared with developed areas [33]. Oral-oral or fecal-oral transmission occurs in early childhood within families, and frequently leads to lifelong persistent infection [10, 26].

Although infected patients may develop chronic active gastritis, most infections are asymptomatic [36, 46]. Infection increases the risk of peptic ulcer disease (PUD), stomach adenocarcinoma, and lymphoproliferative disease of the stomach [29, 51]. Therefore, *H. pylori* was classified as a class I carcinogen in humans by the World Health Organization and International Agency for Research on Cancer [50].

The clinical outcome of *H. pylori* infection has been associated with bacterial virulence factors as well as host and environmental factors [32]. Two virulence factors of

H. pylori, cytotoxin-associated gene A (*cagA* gene) and the vacuolating cytotoxin A gene (*vacA* gene), play a major role in determining the clinical outcome of *H. pylori* infection [9].

The *cagA* gene, the first virulence factor detected in *H. pylori* strains, encodes a protein (CagA protein) that is associated with increased intensity of gastric inflammation and dense neutrophil infiltration [31, 38]. In addition, the CagA protein frequently induces interleukin-8 (IL8) [23] which plays a crucial role in the inflammatory cell response to infection [13]. The *cagA* gene, which is not present in every *H. pylori* strain, is considered to be a marker for a genomic pathogenicity island (*cag*-PAI) [12]. It is considered that this gene with others on the island is correlated with more severe clinical outcomes, including PUD and gastric cancer (GC) [35, 39].

In contrast to the *cagA* gene, the *vacA* gene is present in nearly all *H. pylori* strains around the world [14]. It has been found that the presence of the *cagA* gene is strongly correlated with expression of the vacuolating cytotoxin activity [44]. Furthermore, it has been found that most strains possessing CagA also possess the more virulent vacuolating form of VacA [3]. Although *H. pylori* has a global distribution, geographical differences in the prevalence of *cagA* status among *H. pylori* isolates have been reported [2].

The *cagA* genotype of *H. pylori* can be best identified by molecular methods, using either cultured isolates or direct gastric biopsy specimens. However, this requires invasive endoscopy. Therefore, introduction of accurate serological methods to detect specific antibodies to the *H. pylori* CagA protein would be more suitable for routine clinical use [20]. However, subjects infected with *H. pylori* strains containing the *cagA* gene do not always induce serum CagA antibody [45]. Moreover, it has been suggested that host immunological responses to *H. pylori* may vary in different populations [37].

This study was designed to detect the prevalence of the *cagA* genotype in *H. pylori*-infected Saudi patients with upper gastrointestinal diseases and to investigate its association with severe clinical outcomes, including GC and PUD. Moreover, the study assessed the relationship between *cagA* genotype and the presence of serum anti-CagA antibodies. To the best of our knowledge, this is the first study to examine this relationship in Saudi Arabia.

Materials and Methods

Subjects

The study included 180 adult Saudi patients recruited from those undergoing upper gastrointestinal endoscopy because of dyspeptic complaints or possible gastric cancer in the Gastroenterology & Endoscopy units of King Abdul Aziz specialized hospital and some other hospitals in Taif province and Western region of Saudi Arabia between October 2012 and October 2014. Of these patients, 60 patients had GC (group I), 60 patients had PUD (group II), and 60 patients had non-ulcer dyspepsia (NUD) (group III). NUD patients were defined as those who have dyspepsia without endoscopic lesions of ulcers and/or malignancies [25]. Patients who received non-steroidal anti-inflammatory drugs, antacids, or antibiotics in the 2 weeks prior to examination were excluded. This study was approved by the ethical committees of the hospitals involved and each patient provided a written informed consent before participation in the study.

Specimen Collection

Gastric biopsy specimens. After endoscopic examination, gastric biopsy specimens were obtained from the gastric antrum and examined for the presence of *H. pylori* by the rapid urease test; PyloriTek test kit (Serim Research Corp, USA) and culture. One antral biopsy specimen from each patient was kept in brain heart infusion (BHI) broth (Oxoid, UK) containing 20% glycerol and stored at –70°C until DNA extraction [43].

Blood samples. Blood samples were collected from all patients at the time of endoscopy. Sera were separated and stored at –70°C until tested for detection of the *H. pylori* CagA IgG antibodies.

H. pylori Culture

Gastric biopsy specimens were homogenized and cultured onto *H. pylori* selective agar (Oxoid, UK). Incubation of the inoculated plates was performed at 37°C for 4–7 days under microaerophilic conditions using a gas generator kit (CampGen, Oxoid, UK). *H. pylori* identification was based on colonial and microscopic morphology and confirmed by positive oxidase, catalase, and urease tests. The isolated *H. pylori* strains were preserved at –70°C in BHI broth containing 20% glycerol until DNA extraction was performed [17, 43].

DNA Extraction and PCR for Detection of *cagA* Gene

DNA was extracted from the isolated *H. pylori* strains in culture-positive cases and from gastric biopsy specimens in culture-negative *H. pylori*-infected patients (as indicated by a positive rapid urease test). For patients who were positive for *H. pylori* infection by both rapid urease and culture, DNA extraction and PCR were performed on the isolated strains and the corresponding gastric biopsy specimens. DNA extraction was performed using the QIAamp DNA mini kit (Qiagen, Hilden, Germany), as described by the manufacturer. The extracted DNA was used for detection of the *cagA* gene by PCR using specific primer sets (forward 5′-AAT ACA CCA ACG CCT CCA AG-3′ and reverse 5′-TTG TTG CCG CTT TTG CTC TC-3′) (Macro Gen, Korea), which were designed to amplify a 400 bp fragment from the *cagA* gene. PCR was carried out in a final volume of 25 µl containing 12.5 µl of *Taq* PCR Master Mix (Qiagen), 2 µl (0.5 µg/µl) of template DNA, 1.0 µl (1 µM) of each primer, and 8.5 µl of

Table 1. Demographic characteristics of the studied groups.

	Group I (GC) (n = 60)	Group II (PUD) (n = 60)	Group III (NUD) (n = 60)	P-value
Age (years): mean ± SD	44 ± 6.9	43 ± 2.1	43 ± 9.1	$P1^a > 0.05$ $P2^b > 0.05$
Gender:				
Male	43 (71.7%)	38 (63.3%)	40 (66.7%)	$P1^a > 0.05$
Female	17 (28.3%)	22 (36.7%)	20 (33.3%)	$P2^b > 0.05$
Locality:				
Urban	28 (46.7%)	33 (55%)	30 (50%)	$P1^a > 0.05$
Rural	32 (53.3%)	27 (45%)	30 (50%)	$P2^b > 0.05$
Socioeconomic status:				
Low	18 (30%)	21 (35%)	23 (38.3%)	$P1^a > 0.05$
Medium	22 (36.7%)	23 (38.3%)	19 (31.7%)	$P2^b > 0.05$
High	20 (33.3%)	16 (26.7%)	18 (30%)	

GC: gastric cancer.
PUD: peptic ulcer disease.
NUD: non-ulcer dyspepsia.
[a]Comparison between group I and group III.
[b]Comparison between group II and group III.

PCR-grade distilled water (provided with the *Taq* PCR Master Mix). Amplification was performed with the following program: an initial denaturation step at 94°C for 4 min, followed by 35 cycles, which included denaturation at 94°C for 1 min, primer annealing at 59°C for 1 min, and extension at 72°C for 1 min. Finally, an extension step at 72°C for 10 min was performed. A negative control (without template DNA) was included in each run. The PCR products were separated by electrophoresis using 2% agarose gel run in Tris acetate-EDTA (TAE) buffer and stained with ethidium bromide. The gel was visualized under ultraviolet transillumination. A molecular size marker 100 bp DNA ladder (Cleaver Scientific, UK) was used to determine the size of the bands [8, 19].

Detection of *H. pylori* CagA IgG Antibodies

Detection of *H. pylori* CagA IgG antibodies was performed for the *H. pylori*-infected patients using ELISA kits (MyBioSource, San Diego, CA, USA) according to the manufacturer's instructions. Samples with an antibody index more than 0.9 were considered positive.

Statistical Analysis

SPSS 16.0 software (SPSS Inc., Chicago, IL, USA) was used to analyze the data. The Chi square (x^2), ANOVA test, and odds ratio (OR) were determined. A P-value less than or equal to 5% was considered as significant.

Results

Table 1 demonstrates the demographic characteristics of the studied patients. There was no significant difference regarding age, gender, locality, or socioeconomic status between the group I (GC) and group III (NUD) or between group II (PUD) and group III. *H. pylori* infection was diagnosed in 131 (72.8%) patients by rapid urease test and culture, where 85 patients (47.2%) were positive by rapid urease test, 6 (3.3%) patients were positive by culture, and 40 (22.2%) patients were positive by both rapid urease test and culture. Table 2 shows the *H. pylori* infection status among the different studied groups, where 43 (71.6%) GC patients, 46 (76.7%) PUD patients, and 42 (70%) NUD patients were infected by *H. pylori*.

Table 2. *H. pylori* infection among the studied groups.

H. pylori status	GC (n = 60)	PUD (n = 60)	NUD (n = 60)	P-value
Positive	43 (71.6%)	46 (76.7%)	42 (70%)	$P1^a > 0.05$
Negative	17 (28.3%)	14 (23.3%)	18 (30%)	$P2^b > 0.05$

Positive infection was diagnosed by positive rapid urease test and/or culture.
Negative infection was diagnosed when both rapid urease test and culture were negative.
There was no significant difference regarding *H. pylori* status between group I and group III ($P1 > 0.05$) or between group II and group III ($P2 > 0.05$)
GC: gastric cancer.
PUD: peptic ulcer disease.
NUD: non-ulcer dyspepsia.
[a]Comparison between group I and group III.
[b]Comparison between group II and group III.

Table 3. *cagA* gene and anti-CagA IgG status among *H. pylori*-infected patients in the studied groups.

Tested parameter	Result	GC (n = 43)	PUD (n = 46)	NUD (n = 42)	OR (95% CI)	P-value
cagA gene	Positive	32 (74.4%)	33 (71.7%)	18 (42.9%)	OR1[a] = 3.8(1.5-9.9)	$P1^a < 0.01$
	Negative	11 (25.9%)	13 (28.3%)	24 (57.1%)	OR2[b] = 3.3(1.4-8.3)	$P2^b < 0.01$
Anti-CagA IgG	Positive	30 (69.8%)	33 (71.7%)	18 (42.9%)	OR1[a] = 3 (1.2-7.6)	$P1^a < 0.01$
	Negative	13 (30.2%)	13 (28.3%)	24 (57.1%)	OR2[b] = 3.3(1.4-8.3)	$P2^b < 0.01$

cagA gene status was determined in the 131 *H. pylori*-infected patients.
Sera from the 131 *H. pylori*-infected patients were tested for anti- CagA IgG by ELISA.
GC: gastric cancer.
PUD: peptic ulcer disease.
NUD: non-ulcer dyspepsia.
OR: Odds ratio.
CI: Confidence Interval.
[a]Comparison between group I and group III.
[b]Comparison between group II and group III.

Table 4. *cagA* gene status in the isolated *H. pylori* strains and in the corresponding biopsy specimens.

cagA gene status	*H. pylori* isolates (n = 46)	Biopsy specimens (n = 46)	P-value
Positive	30 (65.2%)	29 (63%)	$P > 0.05$
Negative	16 (34.8%)	17 (37%)	

The *cagA* gene was detected by PCR in the 46 isolated *H. pylori* strains and in the corresponding biopsy specimens.

Detection of the *cagA* gene in the 131 *H. pylori*-infected patients showed that it was positive in 83 (63.4%) patients (32 with GC, 33 with PUD, and 18 with NUD) (Table 3). Fig. 1 shows three positive cases for the *cagA* gene in agarose gel electrophoresis. The *cagA* gene was significantly ($p < 0.01$) higher in patients with GC (74.4%) and PUD (71.7%) compared with those with NUD (42.9%). There was no significant difference between detection of the *cagA* gene in the isolated *H. pylori* strains (65.2%) and its detection directly in the corresponding biopsy specimens (63%) in culture-positive cases (Table 4).

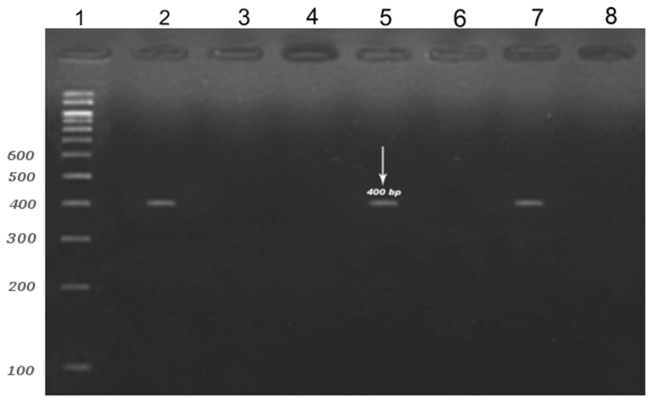

Fig. 1. Agarose gel electrophoresis shows 3 positive cases for *cagA* gene (lanes 2, 5, 7). Lane 1: 100-bp DNA marker, lane 8: negative control.

Table 3 shows that anti-CagA IgG was detected in 81 (61.8%) out of 131 patients who were infected with *H. pylori*. They included 30 patients with GC, 33 patients

Table 5. Evaluation of the role of anti-CagA IgG as a predictor for infection by *H. pylori* strains carrying the *cagA* gene.

Anti-CagA IgG	*cagA* gene status		Total
	Positive	Negative	
Positive	76 (91.6 %)	5 (10.4 %)	81
Negative	7(8.9 %)	43 (89.6 %)	50
Total	83	48	131

The sensitivity of CagA IgG was 76/83 or 91.6%; the specificity was 43/48 or 89.6%; the positive predictive value (PPV) was 76/81 or 93.8%; the negative predictive value (NPV) was 43/50 or 86%, and the accuracy was 76+43/131 or 90.8%.

with PUD, and 18 patients with NUD. It was significantly higher in patients with GC ($P1 < 0.01$) and those with PUD ($P2 < 0.01$) compared with those with NUD. The relation between presence of CagA IgG by ELISA and presence of the *cagA* gene by PCR (as a "true" test result) in the *H. pylori*-infected patients is demonstrated in Table 5. The sensitivity of CagA IgG was 91.6%; the specificity was 89.6%; the positive predictive value (PPV) was 93.8%; the negative predictive value (NPV) was 86%; and the accuracy was 90.8%.

Discussion

In developing countries, most persons harbor *H. pylori*, where the majority acquires infection during childhood. In developed countries, lower prevalence is found owing to better socioeconomic circumstances [2].

This study showed that *H. pylori* infection was diagnosed in 72.8% of the studied patients. The prevalence of infection in our study is comparable to that found in a Saudi study conducted by Abo-Shadi *et al*. [1], where *H. pylori* infection was detected in 64.7% of the gastric biopsy specimens from patients with upper gastrointestinal diseases. The prevalence found in our study lies within the wide range of *H. pylori* infection (50–80%) reported in Saudi Arabia [5]. In contrast, other studies conducted in some neighboring countries reported lower results, such as in an Iraqi study by Kalaf *et al.* [25] and a Palestinian study by Essawi *et al*. [19], where the prevalence of *H. pylori* infection among the studied patients was 48.6% and 44%, respectively. On the other hand, 91% of the studied dyspeptic patients were positive for *H. pylori* in an Egyptian study by Amer *et al.* [2]. This variability in the prevalence among different studies may be attributed to differences in identification methods, different demographic distribution of the organism among various regions, and previous antibiotic consumption [6, 11, 18].

The prevalence of *cagA*-positive strains of *H. pylori* differs in various parts of the world [2]. In this study, the *cagA* gene was detected in 63.4% of *H. pylori*-infected patients. This finding agrees with that reported in a recent study conducted in Riyadh, Saudi Arabia (61.8%) by Marie [30]. On the other hand, a higher prevalence of the *cagA* gene (81.7%) was recently reported in Taif, Saudi Arabia by Kadi *et al.* [24]; however, the latter study was carried out on a small sample size (33 patients). Our finding is also comparable to that demonstrated in some other countries such as Tunisia [7], Egypt [2], Palestine [19], and Iran [21], where the prevalence of the *cagA* gene was 61.6%, 65.2%, 65.9%, and 68.7%, respectively. However, our result was different from that reported in other countries such as Russia, Turkey, Iraq, Cyprus [22, 27, 42], and East to South Asian countries [15, 16, 47], where the reported prevalence of the *cagA* gene was 85%, 78%, 39.2%, 42.5%, and 90%, respectively.

This study showed that there was a relationship between the presence of the *cagA* gene and clinical status, where the gene was significantly higher in patients with GC and PUD than in those with NUD. This result substantiates a role of *cagA* as a marker for increased virulence of *H. pylori*. This finding is supported by other reports from Saudi Arabia [24, 34], and other developing countries [2, 25, 41, 42]. Moreover, studies from Europe and North America reported a significant correlation between the possession of the *cagA* gene and the risk of developing atrophic gastritis, PUD, and GC [35]. On the other hand, conflicting results have been reported in other countries such as Tunisia [7], Iran [21, 40], China [49], and East to South Asian countries [15, 16, 47]. These studies showed no significant association between *cagA* gene and the clinical outcome of *H. pylori* infection. This discrepancy between the different studies regarding the role of the *cagA* gene in severe clinical outcomes can be explained by the possible existence of several distinct forms of the *cagA* gene with an uneven geographic distribution. These differences in *cagA* genotypes may result in differences in virulence among *cagA*-positive *H. pylori* strains; only some forms of the *cagA* gene may be associated with severe gastroduodenal diseases [52].

In our study, we found no significant difference between PCR amplification of the *cagA* gene from *H. pylori* isolates and from the corresponding biopsy specimens in culture-positive cases. This finding was also reported in other studies and suggests that PCR may have a potential value for studying the *cagA* gene directly from biopsy specimens, allowing rapid identification of patients at high risk for developing PUD or GC [28, 43].

Based on the association of the *cagA* gene with severe clinical outcomes found in our study and other studies, identification of the *cagA*-positive *H. pylori* strains is of great importance for management of patients at risk for developing more severe disease. However, molecular detection of the *cagA* gene either in cultured strains or directly in the gastric biopsy specimens requires endoscopy, which is an invasive method. On the other hand, serology can be performed on noninvasively collected clinical samples, making it more feasible for routine clinical use and mass screening. Moreover, it provides the potential to make improvements in the management of *H. pylori* infection in primary care [20, 37]. However, not all persons

infected by *cagA*-positive *H. pylori* strains form anti-CagA antibodies [45]. Therefore, it was important to evaluate the reliability of anti-CagA detection as a predictor for infection by *H. pylori* strains carrying the *cagA* gene in our study. Our results found that the detection of the CagA IgG is a good indicator for infection by *cagA*$^+$ *H. pylori* strains, with an accuracy of 90.8%. Our finding is comparable to that reported in other studies [20, 37]. In contrast, serum CagA antibody was detected in only 43.1–45.5% of Japanese subjects infected by *cagA*$^+$ *H. pylori* strains [48]. Moreover, in other East-Asian countries, a different CagA seropositivity has been reported despite almost all *H. pylori* possessing the *cagA* gene [45]. These conflicting findings may be attributed to the suggestion that host immunologic responses to *H. pylori* may vary in different populations [37].

In conclusion, our study provides additional evidence for a significant association between infection by *cagA* gene-positive strains of *H. pylori* and severe clinical outcomes, including PUD and GC, in Saudi patients. Additionally, there is a good association between detection of the *cagA* gene by PCR and detection of the anti-CagA IgG by ELISA. However, this association needs to be confirmed by a further study that includes a large number of patients from different Saudi regions.

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