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ORIGINAL ARTICLE

Helicobacter Pylori BabA2, CagA and VacA genes; A New Paradigm for Gastric Lesion and Bacterial Carcinogenesis

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ABSTRACT

Background: Helicobacter pylori (H pylori) is a major etiological agent in several gastroduodenal diseases. The most common virulence markers of H pylori are vacuolating cytotoxin (VacA), cytotoxin-associated gene A (CagA), and blood adhesion binding antigen (BabA). The study aimed to investigate the relation of VacA, CagA and BabA2 genotypes of H pylori strain to the endoscopic findings particularly (peptic ulcer or gastric carcinoma) in infected patients.

Methods: A cross sectional study was carried out in the Clinical Pathology Department and Tropical Endoscopy Unit at the Faculty of Medicine, Zagazig University. The study included 100 patients who attended the Endoscopy unit for Upper gastrointestinal endoscopy due to different complains (epigastric pain, heartburn, regurgitation, dyspepsia and vomiting. Two gastric biopsy samples were taken for Rapid Urease Test (RUT) and PCR.

Results: The study showed 62 out of a hundred patients (62%) were H.pylori positive and 50 out of 62 H pylori-positive cases (81%) revealed one or more virulent factors. The positive rate of VacA, CagA and BabA2 among H.pylori positive patients were 73%.23% and 26% respectively. The allelic variant Vac s2m2 was more prevalent (42%) than other alleles. The most predominant genotype combination VacA/CagA 7/50 (14%). VacA was found among 49% of cases of peptic ulcer. A statistically significant difference between VacA, triple gene combination and the clinical outcome. $P<0.05$ was detected.

Conclusions: The studied genes may predispose to severe gastric disorder as a statistically significant difference was found between VacA, triple positive strain and gastric lesions.

Keywords: Helicobacter pylori; CagA; BabA2; VacA; Gastric lesions.

INTRODUCTION

H pylori is a gram-negative, microaerophilic, motile, spiral-shaped bacterium. Nearly 50% of the world's population is infected with H pylori. The prevalence of the disease is higher in developing countries than the developed countries excluding Japan. H pylori is a major causative agent of different gastroduodenal diseases, such as functional dyspepsia, peptic ulcer, gastric cancer and mucosa-associated lymphoid tissue lymphoma. The environmental conditions, host immunological factors and microorganism virulence affect the clinical outcome following infection with this bacterium [1].

Several invasive and non-invasive diagnostic tests are available for the detection of H. pylori and each test has its usefulness and limitations in different clinical situations. Diagnostic tests are usually divided into invasive (endoscopic based) and noninvasive methods. Invasive diagnostic tests include endoscopic image, histology, rapid urease test, culture, and molecular methods. Non-invasive diagnostic tests included urea breath test, stool antigen test and serological [2].

The (VacA), (CagA), and (BabA) are the most common virulence markers of H pylori. The VacA protein induces vacuolation and apoptosis in epithelial cells, as well as immunosuppressive actions in immunological cells. [3].

CagA is a protein with a molecular mass of approximately 125-140 KDa, encoded by the CagA gene that is translocated into gastric epithelial cells by a type IV secretion system, encoded by cag pathogenicity island [4].

CagA-positive H pylori isolates are associated with a higher level of gastric inflammation and damage, compared to CagA negative strains. Many epidemiological studies demonstrated the association between the CagA-positive strains and increased risk of peptic ulcer, gastric atrophy and gastric cancer [5].

BabA mediates adhesion of H pylori to human gastric epithelium. This antigen is encoded by the polymorphic gene called BabA2. Several studies have suggested that BabA plays a key role in severe functional dyspepsia, peptic ulcer and gastric adenocarcinoma [6].

In addition, the combination of VacAs1 and CagA genotypes (type 1 strains) or even the "triple-positive" strains (VacAs1, CagA and BabA2), showed a stronger association with the occurrence of peptic ulcer, intestinal metaplasia and gastric cancer [7].

For these reasons, the current study was conducted to investigate the presence of VacA, CagA and BabA2 genotypes of H pylori isolate and evaluate its relationship with presence of peptic ulcer or gastric carcinoma.

METHODS

This is a cross-sectional study that was conducted in the Clinical Pathology Department and Tropical Endoscopy Unit at Faculty of Medicine, Zagazig University. Hospitals from January 2018 to January 2019.

One hundred patients were enrolled in this study by systematic random sample. They included 48 females and 52 males. The range of age of the enrolled patients was (20-65) years who underwent endoscopy at the Tropical Endoscopy unit at Zagazig University Hospitals due to different complains such as (epigastric pain, heartburn, regurgitation, dyspepsia and vomiting). The patients who suffered from varices and those who were on proton pumps inhibitors medications were excluded from the study. The study patients were informed about the nature and the purpose of the study and written consents were taken from all participants, the study was approved by the research ethical committee of the Faculty of Medicine, Zagazig University. The study was done according to The Code of Ethics of the World Medical Association (Declaration of Helsinki) for studies involving humans. Their data were confidential. Patients involved in the study underwent upper gastrointestinal endoscopy under fasting conditions of six hours, in left lateral position, and under aseptic condition.

Patients were given 5-15 mg of midazolam to attain conscious sedation. Endoscopy was performed by gastroenterologists who examined the stomach and the duodenum with a video endoscope (Olympus Eyes EXERA III CV 190 Tokyo Japan and Pentax EBM 350 Tokyo Japan).

Two gastric biopsy specimens were collected from the antrum and/or the corpus of the stomach from each patient. one piece was examined by rapid urease test for detection of H. pylori and the second piece was placed in sterile saline solution and stored in -80°C for DNA extraction and PCR. Urease activity in gastric biopsies was detected by Kimberly -Clark CLO test Utah USA. REF60480

DNA extraction from gastric biopsy specimens and PCR assay

H. pylori DNA was extracted from gastric biopsies of the study subjects using Thermo Scientific GeneJet Genomi DNA Purification Kit K 0721, K 0722 Waltham USA, following manufacturer guidelines. Briefly, the tissue was cut into small pieces, collected into microcentrifuge tube and then was resuspended in 180 ul of digestion solution,20 ul of proteinase solution was added and the sample was incubated at 56°C, 20 ul of RNase was added and incubated at room temperature for 10 minutes, 200 ul of lysis solution was added and mixed then 50% ethanol. The prepared lysate was added to GeneJET genomic DNA purification column and was centrifuged, wash buffer I was added and then centrifuged for 1 minute at 8000xg. Then wash buffer II was added to the GeneJET genomic DNA purification column and centrifuged. 200ul elution buffer was added to elute the genomic DNA, centrifuged for one minute. DNA was stored at -20°C

H. pylori genome was amplified by PCR. Primer sequences, sizes, and conditions of PCR amplifications of the GlmM gene for detection and confirmation of H.pylori and the virulence genes (VacA, CagA and BabA2) were designed based on published papers with a modification of PCR mixtures and PCR conditions, manufactured by ThermoFisher Scientific kit. Each PCR of GlmM, VacA, CagA,and BabA2 was performed in a total volume of 25 ml containing 2ul genomic DNA from H. pylori from gastric biopsies positive, 12.5 ul thermo scientific Dream Taq Green PCR Master

Mix (Thermo Scientific K1081 lot 00592067 Waltham USA), 8.5 ul nuclease-free water, 1 ul of each forward primer and 1 ul of each reverse primer.

The reaction mixtures were cycled in an automated thermal cycler (Biometra thermocycler, Gene Amp PCR System 2400, Roche Diagnostic System) programmed as follows: Initial denaturation 95°C 10min, denaturation 95°C 30sec, and annealing 55°C 1 min (changed according to the gene), extension 72°C 1 min, final extension 72°C 5 min, 35cycles. The amplified products were visualized by agarose gel electrophoresis [Table 1].

Statistical analysis

All data were collected, tabulated and statistically analyzed using SPSS 20.0 for windows (SPSS Inc., Chicago, IL, USA). Quantitative data were expressed as the mean ± SD & median (range), and qualitative data were expressed as absolute frequencies (number) & relative frequencies (percentage). Percent of categorical variables were compared using Chi-square test. All tests were two-sided. P-value < 0.05 was considered statistically significant (S), p-value ≥ 0.05 was considered statistically insignificant (NS).

RESULTS

The current study is a cross-sectional study that included 100 patients (48 females and 52 males) their ages ranged from 20-65 years with the mean age was 44.55±10.05 years.

Out of the 100 patients, 62 (62%) were H pylori-positive by both PCR and rapid urease test. Upper gastrointestinal endoscopy of the 62 Helicobacter pylori-infected patients showed incompetent cardia in 4 patients; gastritis in 27 patients; peptic ulcer in 27 patients and 4 patients had gastric cancer. There was no statistically significant association (P≥ 0.05) between age and endoscopic findings, while there was a statistically significant association (P<0.05) between sex distribution and endoscopic findings where females were more associated with gastritis.

There was a statistically significant association between both age and sex and PCR test where H pylori PCR positive cases were more associated with older age (Age>47) (P< 0.001**) and more associated with male sex (P< 0.02*). In addition, there were statistically highly significant associations (P< 0.001**) between PCR test and endoscopic findings where -ve PCR was more associated with incompetent cardia [Table 2].

The extracted DNA from gastric biopsy was used for detecting H pylori GImM, then CagA, VacA and BabA2 were analyzed for the 62 helicobacter pylori positive strains by PCR.

Of the 62 H pylori sample tested, 50 had at least one virulence gene and 12 did not show any virulence gene. Vac A single strain was the most predominant detected in 43% 27/62, BabA2 single gene was detected in 4.8% 3/62, The genotype combinations detected were VacA /CagA positive (11 % 7/62), VacA/BabA2 positive (9.6 % 6/62), CagA/BabA2 positive (3.2% 2/62), triple genes positive (8% 5/62) [Table 3].

There was a statistically significant difference between age and the different virulence genes (P< 0.05*) with VacA s1m2 genotype more associated with older age (age>44). with no statistically significant difference between sex and the different virulence genes (P>0.5) [Table 4].

There was a statistically significant difference between VacA and the endoscopic findings (P< 0.05*) with the most VacA positive cases seen in the peptic ulcer group. There was no statistically significant difference between CagA and BabA and different endoscopic findings [Table 5].

VacA single strain was the most predominant one found in 27/50, and the most common combination was VacA/CagA found in 7/50 [Table 6].

There was a statistically significant difference between the triple gene combination (VacA/CagA/BabA2) and the endoscopic findings with gastric cancer [Table 7].

Table 1: Primer sequences and PCR conditions

Genes	Primer sequences	PCR product	Annealing temperature	References
GImM GImM	AAGCTTTTAGGGGTGTTAGGGGTTT AAGCTTACTTTCTAACACTAACGC	294	95°C 10 min, 95°C 30 sec, 55°C 1 min., 72°C 1 min, 72°C 5 min, 35cycles	8
CagA-CagA	5 AATACACCAACGCCTCCA-3 5 TTGTTGCCGCTTTTGCTCTC-3	400	95°C 10min, 95°C 30sec, 55°C 1 min, 72°C 1 min, 72°C	9

Genes	Primer sequences	PCR product	Annealing temperature	References
			5 min, 35cycles	
VacA (s1/s2)	5 ATGGAAATACAACAAACACAC-3 5 CTGCTTGAATGCGCCAAAC-3	259/286	95°C 10 min,95 30sec, 58°C 1 min, 72°C 1 min, 72°C 5 min, 35cycles	9
VacA (m1/m2)	5 CAATCTGTCCAATCAAGCGAG-3 5 GCGTCTAAATAATTCCAAGG-3	570/642	95 °C 10 min, 95°C 30 sec, 60°C 1 min, 72°C 1 min, 72°C 5 min, 35cycles	9
BabA2	5 CCAAACGAAACAAAAAGCGT-3 5 GCTTGTGTAAAAGCCGTCGT	271	95°C 10min.,95°C 30sec, 60 °C 1 min, 72°C 1 min, 72 5°C min, 35cycles	10

Table 2: Relation between PCR test and the endoscopic findings among the studied participants (n=100).

PCR	Endoscopic findings				a χ 2	P value
	Incompetent cardia	Gastritis	Peptic ulcer	Gastric cancer		
	No (%)	No (%)	No (%)	No (%)		
+ve (n = 62)	4 (6.5 %)	27 (43.5%)	27 (43.5%)	4 (6.5%)	19.5	0.0002**
-ve (n = 38)	16 (42.1 %)	8 (21.1%)	13 (34.2%)	1 (2.6%)		
	20	35	40	5		

A Chi square test (χ2) ** highly statistically significant

Table 3: Distribution of the studied virulence genes and their subtypes among the PCR positive patients (n=62).

Genes	No	(%)
VacA only (n=27)		
S1m1	5	8.1
S1m2	4	6.4
S2m1	4	6.4
S2m2	14	22.5
BabA	3	4.8
VacA / CagA (n=7)		
S1m1	4	6.8
S1m2	1	1.6
S2m1	1	1.6
S2m2	1	1.6
VacA / BabA (n=6)		
S1m1	1	1.6
S1m2	0	0.0
S2m1	1	1.6
S2m2	4	6.4
VacA/ CagA/ BabA (n=5)		
S1m1	2	3.2
S1m2	2	3.2

Genes	No	(%)
S2m1 S2m2	1 0	1.6 0.0
CagA /Bab	2	3.2
Negative strains	12	19.3
Total	62	100%

Table 4: Relation between demographic characteristics and virulence genes among the studied patients (n=50).

Variables	Virulence genes						aχ2	P value
	Vac s1m1	Vac S1m2	Vac S2m1	Vac S2m2	Cag A	Bab A		
Age:								
n=44) < median (n=19) > median (n=31)	9 3	1 6	2 5	4 15	4 10	4 12	12.7	0.014*
Sex								
(n=33) Females (n=17)	9 3	5 2	6 1	11 6	10 4	10 6	1.4	0.8
	12	7	7	19	14	16		

A Chi square test (χ2)

Table 5: Relation between Virulence genes and different endoscopic findings among the virulent gene H pylori positive patients (n=50).

Virulence genes	Endoscopic findings				aX2	P value
	Incompetent cardia (n. 2)	Gastritis (n. 21)	Peptic ulcer (n. 24)	Gastric cancer (n. 3)		
	No.	No.	No.	No.		
VacA						
+ve (n=45) - ve (n=5)	2 0	18 (85%) 3(14%)	22 (92%) 2 (8.3%)	3 (100%) 0	7.7	0.04* P1=0.6 P2=0.91 P3=0.04* P4=0.4 P5=0.28 P6=0.43
CagA						
+ve (n=14) - ve (n=26)	0 2	5 (24%) 16(76%)	6(25%) 18(75%)	3(100%) 0	4.6	0.2
BabA						
+ve (n=16) - ve (n=34)	1(50%) 1(50%)	5 (24%) 16(76%)	8(30%) 16(60%)	2(60%) 1 (30%)	2.9	0.4

Table 6: Distribution the different Virulence genes of H.pylori regarding endoscopic findings (n=50).

Variables	Endoscopic findings				Total
	Incompetent cardia	Gastritis	Peptic ulcer	Gastric cancer	
	No (%)	No (%)	No (%)	No (%)	
VacA (n=27) S1m1 S1m2	1 (3.7) 0 0	13 (48.15) 4 3	13 (48.15) 1 1	0 (0.0) 0 0	27

Variables	Endoscopic findings				Total
	Incompetent cardia	Gastritis	Peptic ulcer	Gastric cancer	
S2m1 S2m2	0 1	1 5	3 8	0 0	
BabA (n = 3)	0 (0.0)	2 (66.7)	1 (33.3)	0 (0.0)	3
VacA/CagA (n=7) S1m1 S1m2 S2m1 S2m2	0 (0.0) 0 0 0 0	3 (42.8) 3 0 0 0	3 (42.8) 1 1 1 0	1 (14.4) 0 0 0 1	7
VacA/BabA (n=6) S1m1 S1m2 S2m1 S2m2	1 (16.7) 0 0 0 1	1 (16.7) 0 0 1 0	4 (66.7) 1 0 0 3	0 (0.0) 0 0 0 0	6
VacA/CagA/BabA (n=5) S1m1 S1m2 S2m1 S2m2	0 (0.0) 0 0 0 0	1 (20) 0 0 1 0	2 (40) 1 1 0 0	2 (40) 1 1 0 0	5
CagA/Bab (n=2)	0 (0.0)	1 (50)	1 (50)	0 (0.0)	2
Total	2	21	24	3	50

Table 7: Relation between the combination of different Virulence genes of H. pylori and endoscopic findings (n=50).

Variables	Endoscopic findings				aχ2	P-value
	Incompetent cardia	Gastritis	Peptic ulcer	Gastric cancer		
	No (%)	No (%)	No (%)	No (%)		
VacA/ CagA						
+ve (n=7) -ve (n=43)	0 (0.0) 2	3 (42.8) 19	3 (42.8) 21	1 (14.4) 2	1.6	0.6
VacA/BabA						
+ve (n=6) -ve (n=44)	1 (16.7) 1	1 (16.7) 20	4 (66.7) 20	0 (0.0) 3	4.1	0.2
VacA/CagA/BabA						
+ve (n=5) -ve (n=45)	0 (0.0) 2	1 (20) 20	2 (40) 22	2 (40) 1	11.3	0.01* P1=0.17 P2=0.318 P3=0.27 P4=0.63 P5=0.002* P6=0.007*
CagA /Bab						
+ve (n=2) -ve (n=48)	0 (0.0) 2	1 (50) 20	1 (50) 23	0 (0.0) 3	4.1	0.3

DISCUSSION

The present study was designed to investigate the presence of VacA, CagA and BabA2 genotypes of H Pylori strain from gastric biopsy specimens in patients with upper gastrointestinal diseases and its relationship with clinical status.

This study revealed a highly statistically significant relationship between the age and PCR positive cases, Where H pylori-positive PCR cases were

more associated with older age (above 47 years old) and (P< 0.02 and male sex. On the other hand, a study done by Laura et al [11] showed a lack of statistical significance between H. pylori prevalence and both age and sex, this may return to the differences in geographic distribution, sample size and the age group. In addition, this study shows that there were highly statistically significant associations (P< 0.001**) between PCR test and endoscopic findings as positive cases were associated with gastritis, peptic ulcer and gastric carcinoma and negative cases were associated with normal mucosa. same as a study done by José et al [12] which shows a higher prevalence of gastric ulcer (p = 0.0022) in patients with H. pylori than in H. pylori-negative patients, these results indicated a correlation between the presence of this microorganism and these pathologies

Furthermore, the study showed a statistically significant difference between age and the different virulence genes (P< 0.05*), but there was no statistically significant difference between sex and the different virulence genes (P>0.5), while in a study done by Khansa et al [13] there was no significant relationship between age and VacA gene but, a statistically significant difference between sex and VacA was found may return to different in number, age and sex of the study group.

In our study, the rate of the VacA gene in the H. pylori-positive patients was 72.5%. This rate was less than those reported in studies done in Korea (100%) [14] Ethiopia (90%), and Netherlands (93%) [15] but higher than a study done in Egypt where VacA was 58% [16]. The failure of VacA gene detection in some cases may be due to the genomic diversity of the bacterial point mutations in the conserved genes. In fact, multiple infections contribute to the genetic diversity by inter-strain gene transfer and recombination which include large insertions or deletions and chromosomal arrangements [17].

The present study revealed a higher detection rate of VacA s2m2 genotype which was considered to be a less virulent strain than VacA s1m1 the same as a study done in Egypt by El-Shenawy et al. [18] while in a study done by Park et al. [14], s1m1 was the higher rate (66.2%). In addition, this study showed a statistically significant difference between VacA and endoscopic findings (P<0.05) as most VacA positive cases were in the peptic ulcer group, this finding agreed with earlier studies in North American, Dutch and German populations Strobel et al. [19]. ON contrary no association was observed between VacA genotype and the presence of PUD in a study done by Wang et al [20]. The reason for the conflicting results was likely to relate to geographic diversity in the distribution of VacA genotypes [20].

CagA is considered a marker for the presence of a pathogenicity island of about 40 kb. The presence of an intact cag pathogenicity island is associated with higher interleukin-8 levels and mucosal inflammation [21]

In the present study, CagA H pylori-positive cases were 22% compared to a previous Egyptian study done by El-Shenawy A et al. [18], while The prevalence of CagA in Europe was reported as (66-73%), The majority of studies conducted in Middle Eastern countries (i.e., Turkey, Egypt, Israel and Jordan) reported that CagA genotype varies between 26% and 44% [22]. In Japan, the rate of CagA is very high (90%) (Hussein et al., (23)

In the present study CagA-positive, was the lowest in gastritis, was the highest in gastric cancer. CagA isolates were found in 6/24 (25%) patients with PUD, 3 (100%) out of 3 patients with GC, and 5 out of 21 (23%) patients with gastritis, although the prevalence of CagA has been linked to the clinical presentations No statistically significant difference of CagA could be established with GC and PUD (P-value 0.2). The same result was reported in Egypt by Amer et al. [24] as they detected the p-value as 0.140.

On the other hand, studies from Europe and North America reported a significant correlation between the possession of CagA and the risk of developing atrophic gastritis, peptic ulcer diseases and gastric cancer [25]. This variation may return to that CagA is a polymorphic gene, especially in its terminal region.

BabA, encoded by the BabA2 gene, has previously shown to mediate adherence of H. pylori to the Lewis b blood group antigen on human gastric epithelial cells [26].

In the present study, BabA2 gene was detected in 25.8 % of H. pylori-positive strains, which was close to a study done in Iran by Abadi et al. [27] where BabA2 was 40.6%. Contrary to studies from Asian countries by Mizushima et al. [28] that report a rate of BabA 2 close to 100% in Taiwan.

In Portuguese and Thailand populations, BabA2 is not a biomarker for peptic ulcer disease or gastric. [29] However, for strains isolated from Germany, Turkey, or Northern Portugal, BabA2 expression was associated with the severity of gastric disease

[30], which may be to the allelic variations of the BabA2 gene [31].

The prevalence of BabA2 gene was 5/21 (23% in gastritis, 8/24 (33%) in peptic ulcer and 2/3 (66%) in gastric cancer. Several studies reported the association of this gene with the appearance of severe gastric damage [7]. However, other studies have claimed no association between this genotype and severe pathologies [32]. The present study revealed that there was no statistically significant difference between BabA and different endoscopic findings (table 8).

According to these results, the CagA+/VacA+ genotype was mostly from patients with PUD and gastritis with the predominance of vacAs1m1 genotype, which was consistent with another study in Egypt by El-Shenawy et al. [18] confirm the association between such VacA genotype and severe gastric outcomes. The same finding was reported from Venezuela by Villalobos et al. [33] in which genotype CagA was not observed as a single genotype, but it was associated with VacA s1 m1 in PUD and gastritis, while among the Iranian population VacA s1-m2, CagA-positive strains were predominantly isolated, irrespective of clinical outcomes [34].

Another combination has been observed VacA/BabA2 (6/50) most of them were associated with peptic ulcer patients with the predominance of s2/m2 genotype but no statistical difference was found same to the results reported by Erzin et al. [30]. A significant association between the babA2 and the vacA s1 and clinical outcome was found by Ozbey I and Aygun C [35] in Turkey.

A combination between CagA and BabA2 was found in 1/24 cases of peptic ulcer but no statistically significant difference could be revealed, on other hand, a study done by Oliveira A et al [36], in Brazil found that the presence of both babA2 and cagA was associated with more marked antral inflammation (P <0.05 for both).

The present study showed a statistically significant difference between Triple positive strain VacA/CagA/BabA combination and the endoscopic findings (P< 0.05*) with the most positive cases was in the gastric cancer group, same as studies in Colombia that correlate peptic ulcer disease and gastric cancer with these genotypes [37]. In addition to a study done by Vega et al. [38] a strong association between combined virulent genotypes and PU (p = 0.0001) was found. However, in Mexico, a study by González-Vázquez et al. [39] showed that The triple-positive strains (CagA, VacAs1m1, BabA2) were isolated from patients with gastritis, but a statistical relationship could not have been established, also Chomvarin C et al. [29] in Thailand did not find any specific disease association between H. pylori genotype and the clinical outcome of infection. Both environmental and host factors in association with bacterial characteristics affect the prediction of the severity of the disease.

CONCLUSIONS

Virulence factors of H.pylori (CagA, VacA and BabA) have been revealed to be as early predictors of gastric atrophy and intestinal metaplasia as CagA and triple gene-positive strains in the studied group were more associated with peptic ulcer and gastric carcinoma Studying a larger group of patients is recommended to confirm the study results.

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