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MOLECULAR STUDY OF *HELICOBACTER PYLORI* VIRULENCE GENES *CagA, Hpa* AND *BabA2* IN EGYPTIAN PATIENTS

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ABSTRACT
Objective: The objective of this study was to detect virulence genes of *Helicobacter pylori (H.pylori) cagA, babA2* and *hpa* in gastric biopsies from patients with different stages of gastritis by polymerase chain reaction to correlate the presence of genes with the severity of the diseases.
Method: A total of 80 non repetitive gastric biopsies from antrum of the stomach were obtained from the patients and subjected to study for histological examination, unease activity, culture for *H.pylori*, and polymerase chain reaction studies of virulence genes *cagA, babA2 and hpa*.
Results: The most frequent detected gene by PCR was hpa (66.7%) and followed by cagA and babA2 (61.6%) for each. There was significant association between the three genes (P=0.0001). The study of the association between the virulence gene of *H.pylori* and different clinical symptoms revealed significant association of dyspepsia with c*agA*(P=0.001) *babA2* and *hpa* (P=0.0001), regurgitation with *cagA* and *babA2*(P=0.002),vomiting with *cagA* and *babA2* (P=0.01, P=0.002, respectively) and nausea with *cagA* and *babA2* (P=0.0001, P=0.03, respectively). The virulence genes were detected in gastric ulcer. The degree of inflammation in histopathological examination was also statistically significant associated with the presence of virulences genes *cagA* (P=0.01), *babA2* (p=0.0001) and *hpa* (P=0.0001)
The present study highlights the presence of virulence genes in H.pylori associated with gastric ulcer. The genes cagA, babA2 and hpa are prevalent among the strains affecting the patients. Moreover, these genes are associated with marked clinical and pathological severity. The genes are significantly associated with each other. Further studies are recommended to validate these findings.

Keywords: Gastritis, Genotypes, H.pylori, cagA, babA2, hpa, PCR

ÉTUDE MOLÉCULAIRE DES GÈNES DE VIRULENCE HELICOBACTER PYLORI CagA, Hpa Et BabA2 DANS LES PATIENTS

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Résumé
Objectif: L'objectif de cette étude était de détecter des gènes de virulence de l'Helicobacter pylori (H. pylori cagA), babA2 et hpa dans les biopsies gastriques de patients atteints de différentes étapes de la gastrite la réaction en chaîne par polymérase à corréler la présence de gènes avec la gravité des maladies.
Méthode: un total de 80 biopsies gastriques répétitifs non d'antre de l'estomac ont été obtenus de patients et l'objet d'étude pour l'examen histologique, l'activité, la culture malaise pour H. pylori, et des études de réaction en chaîne de la polymérase de gènes de virulence, cagA babA2 et hpa.
Résultats: Le plus fréquemment détecté par PCR des gènes a été hpa (66,7 %) et suivie par babA et cagA2 (61,6 %) pour chacun. Il y avait une association significative entre les trois gènes (P =0,0001). L'étude de l'association entre les gènes de virulence de H. pylori et différents symptômes cliniques ont révélé une association significative de la dyspepsie cagA avec(P = 0,001) babA2 et hpa (P = 0,0001), régurgitation avec babA et cagA2(P =0,002), avec des vomissements et cagA babA2 (P = 0,01, P = 0,002, respectivement) et des nausées avec babA et cagA2 (P =0,0001, P =0,03, respectivement). Les gènes de virulence ont été détectés dans l'ulcère gastrique. Le degré d'inflammation dans l'examen histopathologique statistique était associé à la présence de gènes de virulence (cagA P =0,01), babA2 (p =0,0001) et hpa (P =0,0001)
La présente étude met en évidence la présence de gènes de virulence associée à H. pylori dans l'ulcère gastrique. Les gènes, cagA babA2 et hpa est très répandue chez les souches affectant les patients.
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De plus, ces gènes sont associés à la mention de la gravité clinique et pathologique. Les gènes sont associés de façon significative avec l'autre. D'autres études sont recommandées pour valider ces résultats.

Mots-clés: gastrite, génotypes, H.pylori cagA,, babA2, hpa, PCR

INTRODUCTION

Helicobacter pylori (*H.pylori*) is spiral shaped gram negative bacilli that is associated with varieties of gastrointestinal disorders that range from mild gastritis up to gastric cancer. This bacterium is acquired during early adulthood, transmitted to family members and in the absence of adequate treatment leads to prolonged life time colonization (1). There is a strong association between the *H.pylori* and gastric cancer that leads to its classification as type 1 definite carcinogen by WHO (2).

H.pylori has several virulence factors that is associated with the severity of the clinical symptoms related to the infection by this pathogen (3). Among the known virulence factors are *ureA, cagA. VacA, dupA, bab* and *SabA. CagA* gene encodes a high immunogenic protein that has been used to identify *H.pylori*. *CagA* interacts with intracellular components of gastric epithelium and leads to its disruption with proinflammatory cytokines secreted due to the infection (4). *VacA* is another virulent gene that induce virulent factor leading to the host cells vaculation. This virulence factor is known to be a multi-receptor protein that leads to membrane depolarization, mitochondrial dysfunction, autophagy, activation of mitogen-activated protein kinases, inhibition of T cell function, and induction of apoptosis. These functions contribute to the persistent colonization of *H. pylori* and its pathogenesis in several upper digestive tract diseases(5) .

CagA gene is another virulent gene that has been studied extensively *as H.pylori* virulent gene (6). *CagA* gene encodes a highly immunogenic protein (7). In Western countries, it has been reported that individuals infected with *cagA*-positive *H.pylori* is at high risk for severe gastrointestinal disorders (8). *CagA* gene is located at one end of the cag pathogenicity island(PAI), that is inserted into *H.pylori* genome from an unknown source (9). *CagA* molecules are directly translocated into gastric epithelial cells by a bacterial type-IV secretion system(T4SS). cagA (10).

Another virulent gene factor is *H.pylori* agglutinin (HPa) that leads to formation of HPa that is a flagellar protein that binds to the surface of gastric mucosal cells. (11). *Hpa* genes code proteins that facilitate bacterial virulence by increasing the production of cytotoxin and cell adhesion to the host cell. The presence of these genes have severe clinical consequences in gastroduodenal patients associated with dyspepsia (11).

Outer membrane proteins (OMPs) are another important virulence proteins. There are several OMPs such as *BabA, SabA, AlpAB,* and *OipA* that have been predicted to play a vital role in adhesion of *H.pylori* to gastric mucosal cells. BabA2 protein is binding factor in *H.pylori*, that is identified by its ability to bind to B-blood type Lewis antigen on the epithelial cells of the stomach (12). The gene that codes BabA2 protein has two alleles babA1 inactive gene and babA2 is the active gene form . (12).

There are evidence that certain combinations of virulence factors such as presence of cagPAI with OMPs leads to virulent strains with severe clinical manifestations (13).

The objective of this study was to detect virulence genes of *Helicobacter pylori cagA, babA2* and *hpa* in gastric biopsies from patients with different stages of gastritis by polymerase chain reaction to correlate the presence of genes with the severity of the diseases.

Methods

The study was carried out in Mansoura University Hospital laboratory. Patients with various types of dyspepsia and subjected to diagnostic endoscopy were recruited from Gastroenterology Center, Mansoura University from January 2017 till January 2018. The study was performed according to the principles of Declaration of Helsinki and approval consents were obtained from each patients.

The inclusion criteria were patients above 18 years, eligible for endoscopy complaining of dyspepsia more than 2 weeks. Patients with history of antibiotics and acute dyspepsia due to drugs intake were excluded from the study.

Samples

A total of 80 non repetitive gastric biopsies from the antrum of the stomach were obtained from the patients and divided into two containers one container with 10% formalin for histopathological examination and the other with sterile normal saline in sterile container for microbiological studies. Samples were rapidly transported to the laboratory.

Histologic Evaluation

Hematoxylin and esosin staining was performed for slides according to the standard techniques of the examination and the grade of the inflammation was reported according to score from 0-3. Geimsa stain was used for detection of *H.pylori* (14).

Rapid Tube Urease Test

The gastric biopsies for microbiological studies were dissected and part of it was inoculated to agar tube to perform rapid unease test as described previously (15). The positive result was indicated by turn of the media to pink color after one hour

incubation at 35° C.

Culture for *H.pylori*

Part of the biopsies in sterile saline was homogenized by sterile glass road and inoculated to Columbia blood agar supplied by 5% of blood and incubated for 10 days under microaerophilic conditions supplied by gas packs (Campy Pak; Becton Dickinson) at 37°C. The colonies were identified by gram stain, positive urease, oxidase and catalase biochemical reactions (16).

Positive samples for H.pylori was interpreted according to positive results of any laboratory tests positive urease, culture and/ or histopathology (17).

Polymerase Chain Reaction for Virulence Genes (PCR)

DNA Extraction

DNA was extracted from part of gastric sample transported in sterile saline that was positive for *H.pylori*. DNA was extracted by mini kit of Qiagen used for tissue DNA extraction according to the manufacturer recommendations (Qiagen extraction kit). The extracted DNA was kept at -20^{o}C till amplification procedures.

Multiplex PCR for amplification of *babA* and *hpa* Genes

The primers sequences used for amplification of *babA* and *hpa* genes were summarized in table 1 (20).

The total volume of the amplification reaction was 50 μL with 5μL DNA template and 5 μL 10X PCR buffer, 2.5 mM Mgcl2, 0.2 mM of each dNTP, 1 μL forward and reverse primer, and 2U Taq polymerase enzyme (Qiagen).

The PCR cycling situations were as follow: original denaturation stage at 95 for 5 minute, 35 cycles of 95°C for 1 minute, 60°C for 1 minute, 72°C for 1 minute, and last extension phase at 72°C for 5 minutes. PCR outcomes were analyzed by 1.5% agarose gel electrophoresis premixed with ethidium bromide and visualized under a UV transilluminator (18).

PCR for *cagA*

The total amount of the amplification mixture was 25 µl with 3 µ of the extracted DNA and 10 pmol of primers of cag5c-F and cag3c-R added to ready to use amplification mixture supplied by Qiagen. Products were amplified using Perkin-Elmer 9700 thermal cycler with the following program denaturation for 3 min at 94°C, 35 cycles of sequential 1 min at 94°C-1 min at 55°C- 1 min at 72°C, and finally 10 min at 72°C. Detection was performed by gel electrophoresis 2% for 20 minutes (19).

Statistical Analysis

Data were collected, revised, coded and entered to the statistical package for social science (SPSS) version 20. The quantitative data were presented as mean, standard deviations and ranges. The comparison between the studied groups were done by using One Way Analysis of Variance (ANOVA). P was considered significant>0.05.

RESULTS

The present study included 80 patients, 42 females (52.5%) and 38 males (47.5%) with mean± age SD 56.1± 10.5 years. The most frequent symptoms were regurgitation (90.0%), abdominal pain (87.5%), vomiting (86.3%) and dyspepsia (83.8%). The most frequent findings of the endoscopic examination was gastric ulcer (50%). The histopathological examination of the gastric biopsies revealed moderate inflammation in 25% of the samples and severe inflammation in 18.8%. *H.pylori* was positive by culture in 45%, by histopathological examination in 45% and by rapid urease test in 41.3%. The total positive samples by any of the used methods was 45%, table 2.

TABLE (1): THE PRIMERS SEQUENCES AND BP OF THE AMPLIFICATION PRODUCTS

Gene	Primer Sequences	bp
bab A2	**5′-AATCCAAAAAGGAGAAAAAGTATGAAA-3′** **5′-TGTTAGTGTGATTTCGGTGTAGGACA-3′**	**832**
hpa	**5′-ATAAAGCTTTCGGTG GTGGTGGAACGATG-3′** **5′-TATCTCGAGTTGTCGGTTTCTTTGC-3′**	**850**
cagA '	**5′-GTTGATAACGCTGTCGCTTC-3′** **5′-GGGTTGTATGATATTTTCCATAA-3′**	**350**

1TABLE (2): DEMOGRAPHIC, CLINICAL AND LABORATORY FINDINGS OF PATIENTS (N=80)

Gender **Male (N0.-%)** **Female (N0.-%)**	 **38 47.5** **42 52.5**
Age (mean SD-years)	**56.1± 10.5**
Dyspepsia(N0.-%)	**67 83.8%**
nausea(N0.-%)	**60 75%**
Abdominal pain (N0.-%)	**70 87.5%**
Vomiting (N0.-%)	**69 86.3%**
Regurgitation (N0.-%)	**72 90.0%**
Floating (N0.-%)	**8 10.0%**
Dyspepsia (N0.-%)	**20 25%**
Gastric ulcer (N0.-%)	**40 50%**
Duodenal ulcer (N0.-%)	**20 25%**
Grade of inflammation **Mild (N0.-%)** **Moderate(N0.-%)** **severe(N0.-%)**	 **15 18.8%** **20 25.0%** **15 18.8%**
Culture (N0.-%)	**36 45%**
Histopathology (N0.-%)	**36 45%**
Rapid urease tube test (N0.-%)	**33 41.3%**
Positive for H.pylori (N0.-%)	**36 45%**

The comparison of the clinical and pathological findings of the patients with *H.pylori* and those without, revealed significant association of dyspepsia (P=0.0001), regurgitation (P=0.001), vomiting (P=0.001), abdominal pain (P=0.0001) and nausea (P=0/003) and presence of *H.pylori*. Moreover, the degree of the inflammation by pathological examination revealed significant association of moderate (44.4%) and severe inflammation in patients with *H.pylor*i compared to those without *H.pylori* (P=0.0001), table 3.

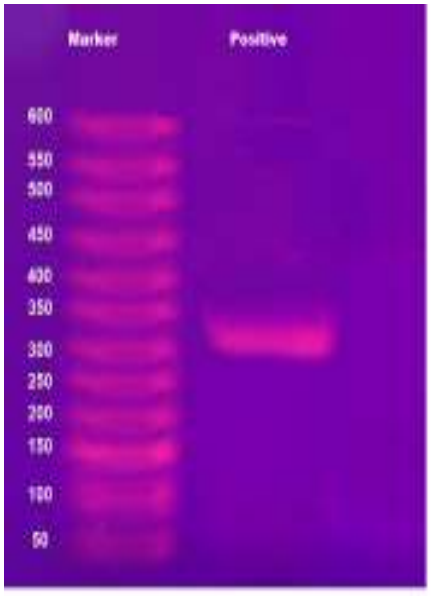

FIGURE (1): PCR POSITIVE FOR CagA GENE COMPARED TO MARKER

TABLE (3): THE COMPARISON OF THE CLINICAL AND PATHOLOGICAL FINDING BETWEEN THE PATIENTS WITH H.PYLORI AND PATIENTS WITHOUT H.PYLORI

	Positive (n=36) No. %	**Negative (n=54) No. %**	**P**
Gender **Male** **Female**	 **20 55.6%** **16 44.4%**	 **18 33.3%** **36 66.7%**	**P=0.2**
Dyspepsia	**24 66.7%**	**5 9.2%**	**P=0.0001**
Regurgitation	**8 22.2%**	**0 0%**	**P=0.001**
vomiting	**10 27.8%**	**1 1.9%**	**P=0.001**
nausea	**9 25%**	**1 1.9%**	**P=0.003**
Abdominal pain	**16 44.4%**	**2 3.7%**	**P=0.0001**
Floating	**6 16.7%**	**2 3.7%**	**P=0.1**
Gastric ulcer	**36 100%**	**0 0%**	**P=0.001**
Grade of inflammation **No** **Mild** **Moderate** **severe**	 **0 0%** **7 19.4%** **16 44.4%** **13 36.1%**	 **28 51.9%** **8 14.8%** **6 11.1%** **2 3.7%**	**P=0.0001**

TABLE (4): THE FREQUENCY OF THE VIRULENCE GENES IN H.PYLORI

Gene	No. %
cagA	22 61.15%
babA2	22 61.6%
hpa	24 66.7%

The most frequent detected gene by PCR was *hpa* (66.7%) and followed by *cagA* and *babA2* (61.6%) for each, Table 4. There was significant association between the three genes (P=0.0001), data not shown.

The study of the association between the virulence gene of *H.pylori* and different clinical symptoms revealed significant association of dyspepsia with *cagA*(P=0.001) *babA2* and *hpa* (P=0.0001), regurgitation with *cagA* and *babA2*(P=0.002),vomiting with *cagA* and *babA2* (P=0.01, P=0.002, respectively) and nausea with *cagA* and *babA2* (P=0.0001, P=0.03, respectively). The virulence genes were detected in gastric ulcer. The degree of inflammation in histopathological examination was also statistically significant associated with the presence of virulences genes *cagA* (P=0.01), *babA2* (p=0.0001) and *hpa* (P=0.0001), table 5.

TABLE (5): THE ASSOCIATION OF DETECTED GENES WITH CLINICAL AND PATHOLOGICAL FINDINGS.

	CagA	BabA2	hpa
Gender Positive Male Female Negative P	 13 9 25 33 0.2	 14 8 24 34 0.1	 12 12 27 29 0.5
Age Positive Negative P	 55.01 7.6 56.2 7.1 P=0.9	 55.6 8.9 56.3 6.4 P=0.7	 54.7 7.5 56.6 6..9 P=0.3
Dyspepsia P	14 P=0.001	15 P=0.0001	15 P=0.0001
Regurgitation P	6 P=0.002	6 P=0.002	5 P=0.05
Vomiting P	7 P=0.01	6 P=0.002	5 P=0.05
Abdominal pain P	5 P=0.1	6 P=0.02	5 P=0.2
Nausea P	12 P=0.0001	9 P=0.03	9 P=0.2
Floating P	 3 P=0.5	 4 P=0.2	 4 P=0.2
Gastric ulcer	22	22	24
Grade of inflammation No Mild Moderate severe	 2 5 7 8 P=0.01	 1 5 6 10 P=0.0001	 0 5 9 10 P=0.0001

DISCUSSION

The endoscopic examination for patients complaining of upper gastrointestinal disorders such as dyspepsia is an essential tool for appropriate diagnosis for differentiation between functional and organic disorders (20).

In the present study endoscopic examination revealed that 50% of the patients had gastric ulcer and 25% had duodenal ulcer and 25% had simple dyspepsia. Previous study reported that simple dyspepsia can be detected in up to 30% of patients upon endoscopic examination (20). Therefore

endoscopic examination and biopsies of patients with dyspepsia is recommend for appropriate diagnosis.
In the present study all gastric ulcer were due to presence of *H.pylori* similar to previous reports (20). On contrary , there were various reports about the decline of *H.pylori* infections in developed countries due to improved sanitation (21, 22). However, in patients with age above 50 years like those in the present study still more affected by *H.pylori* due to earlier exposure.

The virulence genes associated with *H.pylori* also affect the outcome of the infection. The adhesion of *H.pylori* to gastric epithelium leads to symptomatic infections and persistence of infection (4). Among the virulence factors is *babA2* which attach to the blood group antigen Lewis-b present in the gastric epithelium cells. BabA2 was a frequent virulence gene detected in the present study in 61.6% of the positive samples for *H.pylori*. This prevalence was similar to previous reports with range for detection of this gene from 71.6% up t0 82.3% (23, 24) and higher than other report (18).
BabA2 gene is associated with severe inflammatory reactions in gastrointestinal epithelium and it was reported to be a marker to identify patients who will develop severe forms of *H.pylori* associated disease (25). This statement was on line with our findings of the association of babA2 with severe clinical symptoms and pathological findings of moderate to severe pathological scores.

The most prevalent virulence gene detected in the present study was *hpa* gene which was detected in 66.7% of *H.pylori*. This prevalence is similar to that reported by previous study of Heider et al., 2017 (18). *Hpa* gene produce adhesion protein A which is a conserved protein of the surface lipoprotein of *H.pylori* that is essential for adhesion of *H.pylori* and induction of the inflammatory immune response with specific productions of antibodies to it. This ability to produce specific humoral response is being studied and it gives the appeal for *hpa* to be candidate to develop a vaccine for *H.pylori* (26).

Hpa gene was associated also in the present study with severe clinical symptoms, association with the presence of gastric ulcer and with moderate to severe inflammatory scores in the histopathological examination. In previous study *hpa* was the most frequent detected in gastric ulcer, however there was no significant association was reported with the presence of this gene and the severity of the clinical presentation (27). This difference between the findings in the present study and previous might attributed to the finding of the significant association of the different virulence gens present in the present study. So, virulence genes association enhance the pathogenic effects of each other.
The other virulence gene studied in the present study was *cagA* gene. The prevalence of *cagA* gene, another virulence gene of *H.pylori*, was 61.6%. Previous studies reported that the prevalence of cagA gene van reach up to 100% among *H.pylori* strains by different methods of studies either serological or by PCR (28, 29). However, other studies reported a lower prevalence rates (30,31). The prevalence rates of cagA depends upon the geographical location of the isolates.

There was association with gastric ulcer and the presence of *cagA* gene and also significant association of severe clinical symptoms and moderate to severe degree of the inflammation of the histopathology scores. This findings were similar to previous studies (31,32). *CagA* factor is known to be a strong inducer to the pro inflammatory cytokines such interleukin 8 associated with severe inflammatory response in the gastric mucosa (33). CagA also is associated to the risk of gastric cancer development due to H.pylori infection (34).
The present study highlights the presence of virulence genes in H.pylori associated with gastric ulcer. The genes cagA, babA2 and hpa are prevalent among the strains affecting the patients. Moreover, these genes are associated with marked clinical and pathological severity. The genes are significantly associated with each other. Further studies are recommended to validate these findings.

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