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Original article

Detection of *Helicobacter pylori vacA*, *cagA* and *iceA1* virulence genes associated with gastric diseases in Egyptian patients

Ahmed El-Shenawy [a], Manal Diab [a], Mohamed Shemis [b], Maged El-Ghannam [c], Dalia Salem [a,*], Moustafa Abdelnasser [d], Mohamed Shahin [b], Mahmoud Abdel-Hady [d], Effat El-Sherbini [a], Mohamed Saber [b]

[a] *Microbiology Department, Theodor Bilharz Research Institute (TBRI), Giza, Egypt*
[b] *Biochemistry Department, Theodor Bilharz Research Institute (TBRI), Giza, Egypt*
[c] *Gastroentrology and Hepatology Department, Theodor Bilharz Research Institute (TBRI), Giza, Egypt*
[d] *Medical Microbiology and Immunology Department, Faculty of Medicine Al-Azhar University, Egypt*

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ABSTRACT

Background: *Helicobacter pylori* (*H. pylori*) virulence markers would be useful to predict peptic ulcer disease (PUD) or gastric cancer.
Aim: In Egypt, since inadequate data are present regarding *H. pylori* virulence–related genes in different age group patients with gastro-duodenal diseases, it becomes crucial to study the clinical status of *cagA*, *vacA* and *iceA*1 genotypes of *H. pylori* strains recovered from patients with dyspepsia.
Subjects and methods: The study included 113 dyspeptic patients who were exposed to upper gastrointestinal endoscopic examination. Four antral biopsies were obtained from each patient for the analysis of *H. pylori* infection by rapid urease test and detection of *16S rRNA*.
Results: Sixty (53.1%) patients were confirmed to be infected with *H. pylori*. Upon endoscopy, gastritis was revealed in 27 patients (45%) and10 patients (16.7%) had PUD. Of the 60 *H. pylori* strains, 39 (65%) had at least one virulence gene. Six different genotypic forms were recognized; *vacA* (9/60), *iceA1* (1/60), *vacA/cagA* (7/60), *vacA/iceA1* (13/60), *vacA/cagA/iceA1* (8/60) only one of *cagA/iceA type* and we could not detect *cagA*. The overall *vacA*, *iceA1*and *cagA* genes identified were 61.6%, 38.8%, 26.6% respectively, by PCR-based molecular testing. The *vacA* gene status was highly significant related to gastritis patient ($P \leq 0.036$). The *vacA s1m1* and *s2m2* alleles were significantly found in 50% of *H. pylori* infected patients with PUD and with gastritis 57.1% respectively ($P \leq 0.01$).
Conclusion: In conclusion, the main genotype combinations in the studied Egyptian patients were; *vacAs2m2/iceA1*, *vacAs1m1/cagA,* mostly associated with gastritis, and *vacAs1/cagA/icA*, mainly in PUD. The less virulent *(s2, s2m2) H. pylori* genotypes were found in patients aged over 43 years.

1. Introduction

Helicobacter pylori (*H. pylori*) colonizes the gastric mucosa of the human stomach and establishes long-term infection. Dyspepsia is the most common gastrointestinal disorder, and is the most common indication for gastric endoscopy [1]. Infection with *H. pylori* have been proven to be highly associated with gastritis, peptic ulcer disease (PUD) and adenocarcinoma [2,3]. Since 1994, The World Health Organization (WHO) classified *H. pylori* as Class I carcinogen because of its causal relationship to gastric carcinoma [4–6]. The extent and the severity of these associations depend on several elements, such as bacterial virulence factors, age of the host, genetic susceptibility, immune response and environmental factors [7].

H. pylori has a number of virulence factors that play a role in its pathogenicity and influence its colonization and disease severity. CagA, encoded by *cagA* gene is a part and a marker of cagA Pathogenicity Island (PAI). *CagA*-positive *H. pylori* strains are associated with severe inflammation and increased risk of ulcers and cancer in humans. The presence of *cagA* usually coincide with the

Abbreviations: H. pylori, *Helicobacter pylori*; PUD, peptic ulcer disease; WHO, World Health Organization; PAI, Pathogenicity Island; TBRI, Theodor Bilharz Research Institute; DNA, deoxy-ribonucleic acid; PCR, polymerase chain reaction; 16S rRNA, 16 S ribosomal ribonucleic acid; dNTPs, deoxynucleotide triphosphates; GERD, gastroesophageal reflux disease; SPSS, Statistical Package for Social Sciences; Bp, base pair; Lab, laboratory.

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* Corresponding author at: Theodor Bilharz Research Institute (TBRI), EL-Nile street, Warrak EL-Hadar, Imbaba, Giza, P.O. box 12411, Egypt.
E-mail address: drdaliasalem@gmail.com (D. Salem).

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presence of other virulence factors, including *vacA* [8,9]. VacA is an *H. pylori* toxin with multiple cellular effects in different host cell types. Virtually all *H. pylori* strains produce VacA. However, there is significant variation among strains in their capacity to induce cell vacuolization. This variation is attributed to the genetic structural diversity of the *vacA* gene that can assume different polymorphic rearrangements [10]. The initial studies on *vacA* detected two main polymorphic regions; the signal (s)- and the middle (m)-regions. The s- region assumes two forms *s1* or *s2* allele and the m-region encoded the *vacA m1* or *m2* allele. The *vacA* type *s1* strains appear to be more active than s2 strains and are found more frequently in ulcer disease. The *vacA m1* type strains are associated with greater gastric epithelial damage than *m2* strains. The combination of s- and m-region allelic types determines the production of the cytotoxin and is associated with pathogenicity of *H. pylori* strains. *vacA s1/m1* strains produce a large amount of toxin, *s1/m2* strains produce moderate amounts and *s2/m2* strains produce very little or no toxin [11–13].

The *iceA* gene has two main allelic variants; *iceA1* and *iceA2*. The expression of iceA1 is up-regulated on contact between *H. pylori* and human epithelial cells, and may be associated with peptic ulcer disease [14]. However, several studies were not able to explain the role of *iceA* and its correlation with clinical outcomes in other populations; therefore the mechanism of how *iceA* induce PUD remains unclear. Such contradicted results between the *iceA* genotype and clinical consequences could be explicated by the genetic diversity or differences in the geographic region, which were previously reported for other virulence factors [15].

In Egypt, since inadequate data regarding *H. pylori* virulence – related genes in different age group patients with gastroduodenal diseases (gastritis and peptic ulcer disease), it becomes crucial to study the clinical status of *cagA*, *vacA* and *iceA*1 genotypes of *H. pylori* strains recovered from patients with dyspepsia.

2. Subjects and methods

2.1. Patients and clinical specimens

A total number of 113 adult patients undergoing upper endoscopy with various dyspepsia symptoms (abdominal or epigastric pain, vomiting and/or heartburn) at the Endoscopy Unit, Theodor Bilharz Research Institute (TBRI) Hospital from March 2013 to January 2015 were enrolled in this study. Patients who had received non-steroidal anti-inflammatory drugs, as well as antibiotics, H2 receptors antagonists or proton pump inhibitors four weeks prior to the study were excluded. During upper gastrointestinal endoscopy (Olympus X Q40) for examination of the oesophagus, stomach and duodenum, all abnormalities (gastritis, ulceration, erosion and others) were recorded. Four antral biopsies were obtained from each patient. One biopsy was tested for rapid urease test that was performed using rapid urease liquid test kit (Bussero, Milan, Italy), and the other three gastric biopsies were stored in sterile physiological saline in sterile Eppendorf tubes and kept at −70 °C until processed as panel for Deoxyribonucleic acid (DNA) extraction, were used directly for Polymerase chain reaction (PCR) assays. A patient was considered to be infected with *H. pylori* when he had positive rapid urease test and confirmed by detection of *16S rRNA* in gastric biopsy specimens. The protocol was approved by Theodor Bilharz Research Institute (TBRI) institutional review board (FWA00010609) and all patients provided a written informed consent.

2.2. DNA extraction from gastric biopsy specimens and PCR assays

Genomic DNA was extracted from gastric biopsy specimens using (QIAamp DNA Mini Kit (50) 51304 from QIAGEN, USA), catalogue number #51304 following manufacturer guidelines. PCR for *16S* rRNA, *cagA, vacA* and *iceA1* gens were performed in a volume of 50ul with approximately 5 μg of extracted DNA, 200 μM (each) dNTPs, 25 pmol for each primer (Table 1), 1.5 μM Magnesium Chloride and 1unit of *Taq* polymerase (Gotaq Flexi DNA, M8305, Promega, Inc, USA). The reaction was done in PTC-100™ thermal cycler (MJ Research, USA), programmed as follows: denaturing at 95 °C for 5 min, followed by 37 cycles of denaturation at 95 °C for 1 min, annealing at 60 °C for 1 min and extension at 72 °C for 1 min, and final extension at 72 °C for 5 min. [13,16,17].

Each PCR product was separated on 2% agarose gel with ethidium bromide, and 50 bp ladder used as DNA molecular weight standard. In each PCR assay, a negative control (lacking DNA) was include

3. Results

Out of 113 patients included in the study, 60 (53.1%) were confirmed to be infected with *H. pylori*. They presented mostly with dyspepsia 34 (56.7%) followed by vomiting 16 (26.7%) and then heart burn 10 (16.7%).

Upper gastrointestinal endoscopy of the 60 *H. pylori* infected patients revealed gastritis in 27 (45%), whereas 10 (16.7%) had peptic ulcer disease and 4 (6.7%) had normal gastric mucosa. Other endoscopic findings as gastric prolapse, gastroesophageal reflux disease (GERD), oesophageal varices, esophagitis and hiatus hernia were detected in 19 (31.7%) (Table 2).

DNA extracted from gastric antral biopsy specimens was used directly for detection of *H. pylori 16S rRNA*. The *vacA, cagA* and *iceA1* genotypes of *H. pylori* were analysed for the 60 *H. pylori* strains by PCR assays.

3.1. Association between virulence genes and clinical outcomes in 60 H. pylori strains

Of the 60 *H. pylori* strains tested, 39 (65%) had at least one virulence gene and 21 (35%) did not show any virulence gene (Table 2).

In the current study, 48.3% (29/60 cases) of *H. pylori* positive specimens had two or three of the virulence genes examined in this study as evidenced by PCR-based molecular testing.

Six different genotypic combinations can be recognized. Considering the presence of only one genotype marker; *vacA* (15%, 9/60) and *iceA1* (1.7%, 1/60) were confirmed. We could not detect *cagA* as a single genotype.

Other genotype combinations were detected; *vacA+/cagA+*(11.7%,7/60), *vacA+/iceA1+*(21.7%,13/60), *vacA+/cagA+/iceA1+*(13.3%, 8/60) and only one *H. pylori* strain of *cagA+/iceA +* existed in a patient with endoscopic findings other than gastritis and PUD.

Among PUD patients (n = 10); 4 (40%) had *vacA+/cagA+*; 3/4 had *vacA s1m1* genotype and one (1/4) had *s2m2*. The *vacAs1m2+/iceA1 +* and *vacA s1m1+/cagA+/iceA1 +* genotype combination were detected in one (10%) patient each. While for gastritis patients (n = 27); 5 (18.5%) were of genotype *vacAs2m2+/iceA1+*, 5 (18.5%) had *vacA*s1+/*cagA+/iceA1+*; 3 were of *vacA s1m1* genotype and 2 were of *vacA s1m2* genotype.

In the present study, not all the patients in whom gastritis (12/27) and PUD (2/10) were diagnosed were infected with virulent genotype *H. pylori.*

3.2. vacA Genotypes Status

The *vacA* gene was detected in 37 (61.6%) of the 60 *H. pylori* studied strains. The *vacA* s-region and m-region were determined in all 37 *vacA* positive strains. *vacA* (*s1*) allele was found in 17/37