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# Genotyping of *Helicobacter pylori* isolates from Egyptian Patients

**Eman M. Abd El Azeem[1], Abdel-Rahman B. Abdel-Ghaffar[1], Maha Hassan Shokaeir[2], Hend Abdelmoniem Ali[2]**

[1]*Department of Biochemistry, Ain Shams University, Cairo, Egypt*

[2]*Ain Shams University Specialized Hospital, Cairo, Egypt*

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## Abstract

The *cag* Aand*bab*A2genes are the common virulence determinants of *Helicobactor pylori* (*H. pylori*). Acquiring virulent strains of *H. pylori* is associated with increased risk for the development of gastric ulcers or cancer. This study was carried out to investigate the prevalence of cytotoxin associated gene A (*cag*A) and blood adhesion binding antigen (*bab*A2) genotypes of *H. pylori* isolates from Egyptian patients and its correlation with the sensitivity to antibiotics included in triple therapy. Culture of stool specimens was performed in stool samples obtained from 60 patients positive for *H. pylori* antigen in stool. DNA was extracted from *H. pylori* pure cultures. Genotyping was performed by PCR, using specific primers for, *cag*A and *bab*A2 genes. Disc diffusion method was used to determine the sensitivity of cultured bacteria to amoxillin (AMX), metronidazole (MTZ) and clarithromycin (CLM). *cag*A was detected in 34.78% of isolates, while the *bab*7 allele of *bab*A2 gene was detected in 43.47%isolates.13% of strains were genotype A (*cag*A+/*bab*A2+), 21.7% of strains were genotype B (*cag*A+/*bab*A2-), 30.4 % of strains were genotype C (*cag*A-/*bab*A2+) and 34.8%of strains were genotype D (*cag*A-/*bab*A2-). The highest prevalence of sensitivity was for AMX 52.2%, while MTZ was the lowest sensitivity prevalence 30.4%, when compared to the mean sensitivity prevalence of both concentrations of CLR (39.15%). According to these finding Genotyping combination (cagA-/babA2-) is the most prevalent and MTZ is not the optimal choice for the treatment of Egyptian patients and should not be included in the treatment regimens for H. pylori in Egypt.

* **Corresponding Author:** Eman M. Abd El Azeem ✉ emannahas@sci.asu.edu.eg

## Introduction

*Helicobactor pylori* (*H. pylori*), the principal species of the genus Helicobacter, is a common human pathogen, which is responsible for a variety of gastro-duodenal pathologies in both the developing and the developed world (Malnick *et al.*, 2014). Although *H. pylori* infects half of the world's population, only a fraction of colonized individuals develop peptic ulcer and gastric cancer (Abdullah *et al.,* 2009; Malnick *et al.*, 2014;). This fact could be associated either with the genetic diversity of *H. pylori*, or with the particular host genetic background, and/or specific interactions between a particular strain and its host (Hatakeyama, 2006). The clinical outcome following infection with this pathogen has been related to environmental conditions, host immunological factors and microorganism virulence (Khalifa *et al.,* 2010). The triple treatment including proton pump inhibitor (PPI) clarithromycin (CLR) and amoxicillin (AMX) or metronidazole (MTZ) proposed at the first Maastricht conference1 to treat *H. pylori* infection has become universal since it was recommended by all the consensus conferences held around the world. However, the most recent data showed that this combination has lost some efficacy and often allows the cure of only a maximum of 70% of the patients, which is less than the 80% rate aimed and far below what should be expected for an infectious disease (Graham and Fischbach, 2010). *Cag* A and *bab*A are the most commonly studied virulence markers of *H. pylori* and there correlation to high risk of developing peptic ulceration, gastric atrophy and gastric cancer (Johannes *et al.,* 2006).

The *cag*A gene is a marker for the presence of the *cag* pathogenicity island (*cag*-PAI) of approximately 40 kb, *H. pylori* strains possessing *cag*A are associated with a significantly increased risk for the development of atrophic gastritis, peptic ulcer diseases and gastric cancer(Yamaoka, 2010).A type IV secretion system translocates *cag*A protein into gastric epithelial cells, where it is phosphorylated. When this modification occurs, *cag*A affects various cellular processes and signal transduction pathways, such as disruption of tight and adherent junctions that lead to pro-inflammatory and mitogenic responses effects (Wen and Moss, 2009).

The blood group antigen binding adhesin (*bab*A) is one of the major outer membrane proteins (78-kDa) of *H. pylori* encoded by the *bab*A2 gene, binds to the fucosylated Lewisb blood group antigens on the gastric epithelium and plays a key role in facilitating bacterial colonization to the stomach (Wen and Moss, 2009). Studies in Western populations have associated the presence of the *bab*A2gene with gastric cancer (Torres *et al.,* 2009). The present study aims to investigate the prevalence of *cag*A and *bab*A2 genotypes of *H. pylori* isolates from Egyptian patients and its correlation with the sensitivity to antibiotics amoxillin (AMX), metronidazole (MTZ) and clarithromycin (CLM) that included in triple therapy.

## Materials and methods

*Patient samples*

A total of 60 stool samples from *H. pylori*-positive patients (25 men and 28 women and 7 Child) were tested for *Helicobacter pylori* antigen in stool presence. History of abdominal pain, heartburn and associated diseases were obtained from patient profile. Patients were included after getting the informed consent as per the criteria laid down by the Institutional Ethics Committee.

*Microorganism culture*

Stool specimens obtained from all patients were processed, diluted and inoculated into the Brain heart infusion agar (*Biolife italiana.*) plates with 2, 3, 5 triphenyletetrazoliumchloride (40 mg/liter) (Algomhoria, Egypt) and Skirrow, s supplement (LabM Limited, UK) and grown under microaerophilic conditions at 37°C for 3 to 7 days. All *H. pylori* isolates were negatively stained with Grams stain and positive for catalase and urease testing.

*DNA extraction and cagA and babA2 genotyping*

Genomic DNA was extracted by CTAB methodology with phenol/chloroform and isopropanol (Xu *et al.*, 2003).Purified DNAs were stored at -20°C until use. In all cases, PCR amplification was carried out in a 25 µL reaction mixture containing 12.5 µl GoTaq® Green Master Mix (Promega), 2 µl sense and antisense primers, and 120 ng genomic DNA.

The PCR had an initial step at 94°C for 1 min, followed by 35 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 1 min, and a final extension at 72°C for 5 min, using AmP PCR system 9700 (Applied biosystems). The primers used and their details are shown in Table 1. Primers to the *glm* Mgene of *H. pylori* were used to control DNA specificity. PCR products were analyzed on 1.5% agarose gel electrophoresis with ethidium bromide. Bands of PCR products and DNA marker determined by ultraviolet illumination.

*Antibiotics susceptibility test*

Susceptibility patterns of *H. Pylori* isolates to MTZ, AML and CLR were determined, concentrations of antibiotics added were 0.12 mg/L for AMX, 0.25 and0.5mg/L for CLR and 8mg/L for MTZ and added to brain heart infusion agar containing Skirrow, s supplement.

Parts of bacterial colonies were spotted on the plates. The discs of antibiotics with desired concentrations were loaded on the surface of inoculated agar and incubated for 72h under microaerophilic conditions. Bacterial colonies able to grow around discs of antibiotics considered as resistant while those couldn't able to grow considered as sensitive. The results were correlated to *cag*A and *bab*A2 status.

**Results**

*Detection of H. pylori genotypes*

All successful cultures (23 strains) were stained and examined microscopically then identified biochemically by examination of catalase and urease activities. Only Gram negative isolates gave positive results for both catalase and agar based urease tests were undergoing further PCR amplification for genotyping and antimicrobial susceptibility test.

**Table 1.** Primers Sequence.

| Primer | Sequence | |
|---|---|---|
| *glm*MF | 5'-CCCTCACGCCAT*CAG*TCCCAAAAA-3' | Paniagua *et al.*,2009 |
| *glm*MR | 5'-AAGAAGTCAAAAACGCCCCAAAAC-3' | 417 bp |
| *cag*F1 | 5'-GATAA*CAG*GCAAGCTTTTGA-3' | Ben Mansour *et al.*,2010 |
| *cag*B1 | 5'-CTGCAAAAGATTGTTTGG*CAG*A-3' | 349 bp |
| *bab*7-F | 5'-CCAAACGAAACAAAAAGCGT-3' | Sheu *et al.*,2003 |
| *bab*7-R | 5'-GCTTGTGTAAAAGCCGTCGT-3' | 271bp |

DNA integrity and specificity was confirmed by *glmM* PCR, which rendered the expected 417 bp band from all isolates (Fig. 1).Amplification of the *cagA* gene was visualized as a band of 349 bp (Fig. 2) using*cag*F1/B1 primers. Also amplification of *bab*A2 F/R primers was visualized as a band of 271bp (Fig. 2).

*Combinations of cag*A *and bab*A2 *genotypes*

Data represented in table (2) showed that, isolates were subdivided into four groups on the basis of their genotype.

The prevalence of *cagA*+/babA2+ genotype (group A) was 13% (3/23), *cagA* +/*babA2*- (group B) was 21.7% (5/23), *cagA*-/*babA2* + (group C) was 30.4 % (7/23) and *cagA*-/babA2- (group D) was 34.8 % (8/23).

**Table 2.** Combination between *cag*A and *bab*A2 genes in 23 Egyptian strains.

| Group | Genotype combinations | Prevalence % |
|---|---|---|
| A | *cag*A+/ *bab*A2 + | 13% (3/23) |
| B | *cag*A+/ *bab*A2- | 21.7% (5/23) |
| C | *cag*A- / *bab*A2+ | 30.4% (7/23) |
| D | *cag*A- / *bab*A2- | 34.8 % (8/23) |

The total prevalence of *cagA* genotype of *H. pylori* was 34.7% (group A and B) while, the prevalence of babA2 genotype was 43.4% (group A and C).

*Relationship between drug sensitivity and cagA and babA2 virulence gene combinations*

In table (3): Analysis of the drug sensitivity indicates the highest prevalence was for AMX sensitivity 52.2% (12/23) while MTZ was the lowest prevalence 30.4% (7/23) when compared to the mean prevalence of both CLR concentrations which was (39.15%).

**Table 3.** Prevalence of antibiotics' sensitivity of each group of genotype combinations.

| Antibiotic | Total Prevalence | Group A, N=3 | Group B, N=5 | Group C, N=7 | Group D, N=8 |
|---|---|---|---|---|---|
| | | n Prevalence % | n Prevalence % | n Prevalence % | N Prevalence % |
| AMX | 52.2(12/23) | 3 3/3(100) | 5 5/5 (100) | 1 1/7 (14.3) | 3 3/8 (37.5) |
| MTZ | 30.4(7/23) | 2 2/3 (66.6) | 1 1/5 (20) | 1 1/7 (14.3) | 3 3/8 (37.5) |
| CLR( 0.25mg) | 52.2(12/23) | 2 2/3 (66.6) | 4 4/5 (80) | 2 2/7 (28.5) | 4 4/8 (50) |
| CLR(0. 5mg) | 26.0(6/23) | 2 2/3 (66.6) | 2 2/5 (40) | 1 1/7 (14.3) | 1 1/8 (12.5) |

n: the number of isolates sensitive to the antibiotic.

**Discussion**

*Helicobacter pylori* has been identified as a major cause of peptic ulcer disease (PUD) and a risk factor for gastric cancer (GC) and mucosa-associated lymphoid tissue (MALT) lymphoma. On a global scale, GC is the second commonest cancer in the world. There is substantial international variation in GC incidence with the highest rates in China and Japan. Epidemiological studies have proved that *H. pylori* infection is considered as a risk factor for gastric cancer, therefore WHO International Agency for Research on Cancer has classified *H. pylori* as a carcinogen. Although the majority of the *H. pylori* infected patients develop no significant clinical disease, some develop two kinds of clinical outcomes, PUD and GC. The reasons may be related to differences in genetic susceptibility of the host, environmental factors, and genetic diversity of *H. pylori* (Saxena *et al.*, 2011).

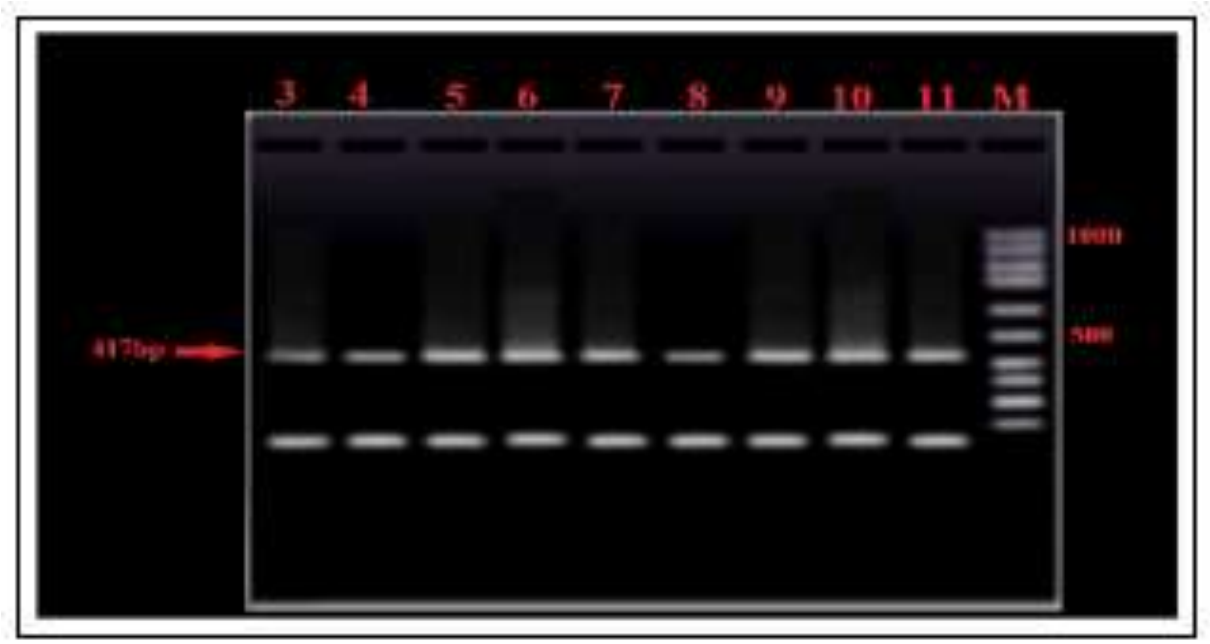


**Fig. 1.** PCR amplification of 417 bp of *glm*M gene of *H. pylori* isolates on 1.5% agarose gel, Lane 10 (M): molecular weight marker (100bp), Lane 1-9 Positive results for *glm*M gene.

In the current study, the prevalence of cytotoxin associated gene A (*cag*A) was (34.7%) this result is in agreement to previous report from Egypt (Hussein *et al*., 2008) and lowers than the studies from Turkey (71.4%) (Ozbey and Aygun, 2012), Kuwait (53%) (Al Qabandi *et al.,* 2005), Iraq (71%) and Saudi Arabia (52%) (Hussein *et al.,* 2008).

Egyptians had a modest positivity of *cag*A prevalence (Al Qabandi *et al.,* 2005) and lower than prevalence reported from Western countries e.g. USA, 60% (Kusters *et al.,* 2006) and England 68%(Go and Crowe 2000).

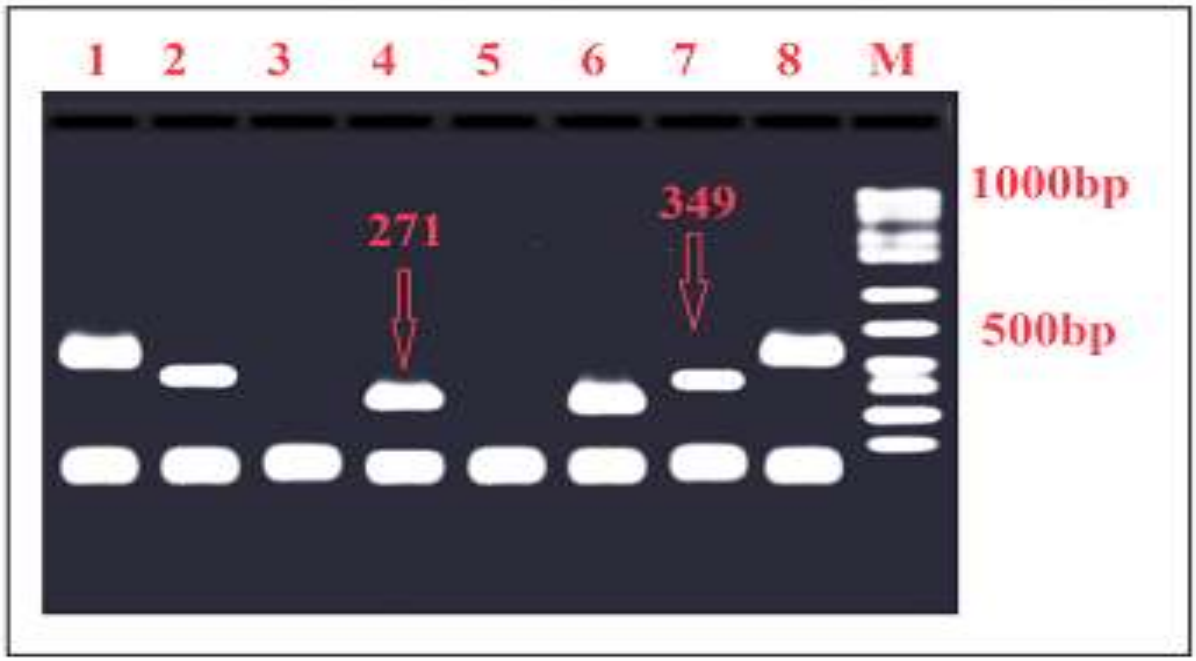


**Fig. 2.** Genotyping of virulence factor genes in Egyptian *H. pylori* isolates. The image shown a representative gel electrophoresis of independent PCR amplification products of *glm*M, *cag*A and *bab*7 allele of *bab*A2 genes.lanes1,8 are bands of 417bpof *glm*M gene; lane 2,7 are bands of 349bp of *cag*A positive strains; lanes 4,6 are bands of 271bp of *bab*7 allele of *bab*A2 positive strains; lane3,5 are negative results of *cag*A gene; M is 100bp DNA ladder.

*cag*A has been implicated as major virulence factor of *H. pylori*. Since there is increasing evidence that genetic variability of *H. pylori* may have clinical importance(Diab *et al*., 2016).*H. pylori* has a global distribution and several reports have evidenced geographical differences in the prevalence of *cag*A status (Amer *et al.,* 2013). *Cag*A prevalence in middle east ranges between 26-76%, in Europe ranges between 66-73%, in America it ranges between 57-75% and in East Asia ranges between 90-100%. *bab*A2 prevalence In Europe ranges between 34-72%, in America it ranges between 46-69% and in East Asia ranges between 80-100% (Torres *et al*., 2009).

Previous studies indicated that the *cag*A-positive strains are directly associated with acute gastritis, gastric ulcer, and gastric cancer development (Matos *et al.,* 2013 and Hatakeyama, 2014).

Antigen binding adhesion *Bab*A2attaches *H. pylori* to epithelial cells, allowing delivery of *cag*A toxins near the gastric epithelium enhancing gastric tissue damage (Yamaoka, 2010). In the present study, Egyptian *H. pylori* isolates exhibited a moderate frequency (43.47%) of the *bab*A2allele corresponding to that reported in Europe which ranges between 34-72% and lower than that reported in America, in the mid-western USA and in Italy, *bab*A2 was positive in only 36% of *H. pylori* isolates (Mattar *et al.,* 2005), and lower than that in China that the *bab*A2-positive strains were found in 79.8% of the patients and were associated with higher lymphocytic infiltration, presence of glandular atrophy, and intestinal metaplasia in the antrum (Yu *et al.,* 2002).

A series of studies of the association between *bab*A2 gene and peptic ulcer diseases and gastric cancer have been performed, but with inconsistent or conflicting conclusions (Mottaghi *et al.,* 2014).

*H pylori* is a globally important and genetically diverse gastric pathogen that infects most people in developing countries. Eradication efforts are complicated by antibiotic resistance, which varies in frequency geographically (Ousman *et al.,* 2013).

In this study Egyptian *H pylori* isolates classified into 4 groups: group A (*cag*A+/*bab*A2+), group B (*cag*A+/*bab*A2-), group C (*cag*A-/*bab*A2+) and group D(*cag*A-/*bab*A2-). The most prevalent genotype combination was group D (*cag*A-/*bab*A2-) with percent 34.8%. Both groups A and B, where *cag*A is positive, possess highest sensitivity prevalence to AMX (100%). Group C possesses the lowest sensitivity prevalence to all tested antibiotics AMX, MTZ and CLR. Analysis of the drug sensitivity indicates the highest prevalence was for AMX sensitivity 52.2%.

The prevalence of sensitivity of Egyptian isolates for CLR (0.25 mg/l) was 52.2% higher than their sensitivity for CLR (0.5mg/l) was 26.1%. The lower prevalence of sensitivity to CLR 0.25mg/l was for *cag*A-/*bab*A2+ combination 28%, while MTZ was the lowest prevalence (30.4%) compared to the mean prevalence of both CLR concentrations (39.15%). Our results are in accord with other reports of many MTZ resistant strains elsewhere in Africa (Senegal, Nigeria, South Africa and Cameroon) (Ndip *et al.,* 2008; Seck *et al.,* 2009; Tanih *et al.,* 2010).

Resistance to useful antimicrobials, especially MTZ and CLA, has been a major problem in some societies, even among people not previously treated for their *H. pylori* infections. Such resistance is generally attributable to inadvertent *H. pylori* exposure during treatment for other conditions (Rimbara *et al.,* 2011). However, the widespread use of metronidazole in Egypt for treatment of numerous infectious diseases likely led to the development of resistance to the drug. With the exception of metronidazole, *H. pylori* isolates were highly sensitive to antimicrobial drugs tested in the current study.

In conclusion, this study has shown moderate prevalence of both *cag*A and *bab*A2 virulence genes in Egyptian *H. pylori* isolates, which is lower than that found in America, East Asia and some Arabian countries. Consequently the presence of more virulent genotype and two positive strains was low in Egyptian patients and decreased risk of developing gastric cancer. On the other hand, this study considered that metronidazole is not the optimal choice for the treatment of Egyptian patients and should not be included in the treatment regimens for *H. pylori* in Egypt, something of great importance is to test the sensitivity prior to applying the treatment and to increase the social awareness about the danger of antibiotic abuse and its long run effects of increased resistivity of microbes and generating new resistant microbes and treatment failure as well.

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