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Helicobacter pylori vacA genotyping in relation to *cagA* status, ultra-structure of gastric mucosa and clinical outcomes in Egyptian patients

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***Helicobactor pylori (H. pylori)* has been strongly associated with gastritis, peptic ulcer and is linked to an increased risk of gastric cancer. The cytotoxin-associated gene product (*cagA*) and the vacuolating cytotoxin (*vacA*) have been implicated as two major virulence factors of *H. pylori*. Since there is an increasing evidence that genetic variability of *H. pylori* may have clinical importance, we aimed to evaluate different *vacA* genotypes and reveal its relationship with endoscopic and transmission electron microscopy (TEM) findings among *H. pylori* infected Egyptian patients. Forty *H. pylori* infected patients possessing *vacA* gene who underwent upper endoscopic examination were considered to be infected with *H. pylori* when rapid urease test and detection of 16S rRNA in gastric biopsy recorded positive. Both *vacA* and *cagA* genotypes were detected by polymerase chain reaction (PCR). The TEM was performed to assess the ultra-structure of the gastric mucosa. Four *vacA* genotypes were identified, the most prominent was the *s2/m2* allele combination (52.5%) followed by *s1/m1* (27.5%), *s1/m2* (17.5%) and *s2/m1* genotype was found just in one *H. pylori* strain (2.5%). There were significant correlations between *vacA s2/m2* and gastritis (65.2%), and *vacA s1/m1* and peptic ulceration (57%). The *cagA* gene was associated with 38% of *vacA* genotypes and 60% of which were significantly associated with *vacA s1/m1* genotype with the development of severe gastritis reaching up to gastric ulcer. The TEM revealed *H. pylori* spiral and coccoid forms, cytoplasmic vacuolar degeneration caused by *vacA*, swollen mitochondria and dilated rough endoplasmic reticulum. In Egypt where prevalence of *H. pylori* infection is high, genotyping of *H. pylori* virulence factors can help to predict patients who are at a high risk of related gastroduodenal diseases. Although *H. pylori* with *vacA s2/m2* genotype is mostly related to low level of virulent strains yet, significant crosstalk between *H. pylori* strains harboring both *vacA s1/m1* and *cagA* gene provides crucial insights into virulence of high level.**

Key words: *Helicobactor pylori*, *vacA* genotyping, *cagA*, gastritis, peptic ulcer.

INTRODUCTION

Helicobacter pylori is a spiral, Gram-negative bacterium that inhabits the stomachs of approximately half of the world's population (Warren and Marshall, 1983). Infections with *H. pylori* may induce gastritis, gastric and

duodenal ulcers and even is linked to an increased risk of gastric cancer (Khalifa et al., 2010). *H. pylori* secrete many of the proteinaceous factors that are important for initial colonization and subsequent persistence in the stomach. Two major virulence factors of *H. pylor*i have been described; the cytotoxin-associated gene product (*cagA*) and the vacuolating cytotoxin (*vacA*). Both play a crucial role in determining the clinical outcome of *H. pylori* infection and their genes could serve as epidemiological markers (Marie, 2012).

The VacA toxin binds to target cells and is internalized causing severe “vacuolation” that has been attributed to the formation of VacA anion selective channels in membranes. In addition to the induction of vacuolation, VacA exerts a variety of other effects on target cells, including disruption of mitochondrial functions, stimulation of apoptosis and blockade of T-cell proliferation *(*Palframan et al., 2012).

The *vacA* gene, encoding the vacuolating toxins is virtually present in all strains of *H. pylori*. Polymorphism in *vacA* gene sequence has been identified in three variable regions; signal (s) region, mid (m) region and intermediate (i) region. Two types of allelic variations in the s-region and m-region are classified as s1 or s2 and m1 or m2 respectively. Therefore, the *vacA* gene of a given strain is composed of any of the possible combination of s and m region sequence type *(*Atherton et al., 1995). Type *s1/m1 vacA* causes more epithelial cell damage than type *s1/m2,* whereas type *s2/m2* and the rare *s2/m1* are non-toxic (Letley et al., 2003; Argent et al., 2008). In our study we aimed to evaluate different *vacA* genotypes and to reveal its relationship with endoscopic and TEM findings among *H. pylori* infected Egyptian patients.

MATERIALS AND METHODS

Patients and specimens

Forty *H. pylori* infected patients possessing *vacA* genes who underwent upper endoscopy for various dyspeptic symptoms at Endoscopy Unit, Theodor Bilharz Research Institute (TBRI) Hospital in-between March 2012 to April 2013 were enrolled in this study. The mean age of the patients was 50.05 years (range, 17-76 years), 29 were males and 11 were female. None of the patients had received non-steroidal anti-inflammatory drugs, as well as antibiotics, H2 receptors antagonists or proton pump inhibitors in the past four weeks prior to the study. Thorough endoscopic examination of the oesophagus, stomach and duodenum and clinical condition of the patient (gastritis, peptic ulceration, normal endoscopy and other findings) were assessed. Four antral biopsy specimens were obtained from each patient. This study was approved by TBRI Institutional Review Board (IRP) FWA 00010609 and informed consent was obtained from each subject before endoscopic examination. A patient was considered to be infected with *H. pylori* when rapid urease test and detection of 16S rRNA in gastric biopsy specimen were recorded positive.

Rapid urease test for *H. pylori* infection

One gastric biopsy was inserted and completely immersed in the reagent solution which is composed of urea, phenol red and stabilizers (Bussero, Milan, Italy). The positive sample turns color from yellow to magenta within 30 min.

DNA extraction

Two antral gastric biopsy specimens for each patient were stored in 0.9% saline at –70°C until used for polymerase chain reaction (PCR) assay. DNA for PCR was extracted directly from antral gastric biopsy specimens using the QIAamp tissue kit provided by (Wizard SV Genomic DNA Purification System, USA) according to the manufacturer's instruction. The DNA extracts were stored at –20°C until used for PCR assays.

PCR-based *H. pylori* detection, *vacA* genotyping and *cagA* status

PCR assays were performed in a volume of 50 µl with approximately 10 µg of total extracted DNA. PCR amplifications were carried out in Gene Amp PCR system 9700 (Bio Rad T100 thermal cycler). The 50µl reaction mixture consisted of 10 ug of the extracted DNA, x1 PCR buffer, 1.5 mM Magnesium Chloride, 200 µM of each dNTP, 20 pmol of each primer (Table 1) and 1U Taq DNA polymerase (Promega).

PCR amplification of the *H. pylori* 16S rRNA

Amplification was carried using the following cycling parameters: An initial denaturation at 95°C for 5 min and 35 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 30 s. This was followed by a final extension of 72°C for 10 min (Chisholm et al., 2001).

PCR amplification of the *H. pylori vacA* genotypes

The following cycling parameters were used: an initial denaturation at 95°C for 4 min and 35 cycles of 95°C for 1 min, 52°C for 1 min and 72°C for 1 min. This was followed by a final extension of 72°C for 10 min (Falsafi et al., 2009).

PCR amplification of the *H. pylori cagA* gene

The following cycling parameters were applied: an initial denaturation at 94°C for 5 min and 35 cycles of 94°C for 1 min, 59°C for 1 min and 72°C for 1 min. This was followed by a final

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Table 1. Primer sequences and size of expected amplicon of each PCR assay.

Target genes	Primers sequences (5'- 3')	Size of Amplicon (bp)	References
16S rRNA	F: CTG GAG AGA CTA AGC CCT CC R: ATT ACT GAC GCT GAT TGT GC	110	Chisholm et al., 2001
cagA	F:AATACACCAACGCCTCCA R:TTGTTGCCGCTTTTGCTCTC	400	Falsafi et al., 2009
vacA:			
vac A (s)	F: ATGGAAATACAACAAACACAC R: CTGCTTGAATGCGCCAAAC	*s1*:259 *s2*:286	Falsafi et al., 2009
vac A (m)	F:CAATCTGTCCAATCAAGCGAG R: GCGTCTAAATAATTCCAAGG	*m1*:570 *m2*:642	

extension of 72°C for 10 min (Falsafi et al., 2009). Each PCR product was separated on a 2% Agarose gel and 50 bp ladder was used as DNA molecular weight standard. In each PCR assay, negative control (lacking DNA) was included.

Transmission electron microscopy (TEM) examination of gastric mucosa

Segments from each gastric biopsy specimens were collected from *H. pylori* infected patients. Segments were immediately fixed for 2 h in equal volumes of glutaraldehyde 4% and caccodylate 0.2 M. The fixed segments were washed in equal volumes of Sacchrose 0.4 M and caccodylate 0.2 M for 2 h and incubated in osmium tetroxide 2% and caccodylate 0.3 M for 1 h. The samples were washed with distilled water and finally dehydrated in ascending grades of ethyl alcohol for 5 min each (30, 50, 70 and 90%) then absolute alcohol 100% for 10 min for three times. Substitution in a mixture of epoxy resin and ethyl alcohol in equal volumes for 1 h was done. Impregnation in pure resins using Epon A and Epon B in equal volumes making three washes on three successive days was done. The specimens were embedded in epoxy resin to which was added an accelerator DMP 30 in special capsules then left in oven at 60°C for 2 days to polymerize and harden. The thin sections were stained with uranyl acetate and lead citrate and were examined with TEM to assess the interaction between *H. pylori* and the ultra-sructure of the gastroduodenal epithelial cells (Bai et al., 2010).

Statistical analysis

Results are expressed as number (%). Comparison between categorical data was performed using Chi square test. Statistical Package for Social Sciences (SPSS) computer program (version 19 windows) was used for data analysis. P value ≤ 0.05 was considered significant and < 0.01 was considered highly significant.

RESULTS

H. pylori infection in all patients was confirmed by rapid urease test and detection of 16S rRNA of *H. pylori* by agarose gel electrophoresis (110 bp) in gastric biopsy specimens (Figure 1).

Clinical outcomes

During upper gastrointestinal endoscopy of the studied 40 *H. pylori* infected patients possessing *vacA* genes, their gastroduodenal mucosa had developed gastritis in 23 (57.5%), whereas 7 (25%) had peptic ulceration (including one with suspected malignant ulcer). Other endoscopic findings as; oesophageal varices, gastro-oesophageal reflux, gastric prolapse, hiatus hernia were revealed in 9(22.5%) cases and only one patient had normal gastric mucosa.

vacA genotyping

The *vacA* s- and m- region genotype were determined in all the studied 40 *H. pylori* strains. The *vacA s1* allele was found in 18 (45%) of the *H. pylori* strains and the *s2* allele was found in 22 (55%) (Figure 2, Table 2). Whereas *vacA m1* allele was found in 12 (30%) of the *H. pylori* strains and the *m2* allele was found in 28 (70%) (Figure 2, Table 2). Four *vacA* genotypes were identified in the study, the most prominent one was the *s2*/*m2* allele combination (52.5%) followed by *s1*/*m1* (27.5%), *s1*/*m2* (17.5%) and *s2*/*m1* genotype was found just in one *H. pylori* strain (2.5%) (Figure 2, Table 3).

Relationship between *vacA* genotypes and clinical outcome

Upon endoscopy, 15 out of 23 *H. pylori* infected patients with gastritis (65.2%) were significantly associated with *vacA* genotype *s2/m2* allele combination (P< 0.01), whereas in peptic ulceration, 4/7 (57%) of them were significantly associated with *vacA s1/m1* genotype (P<

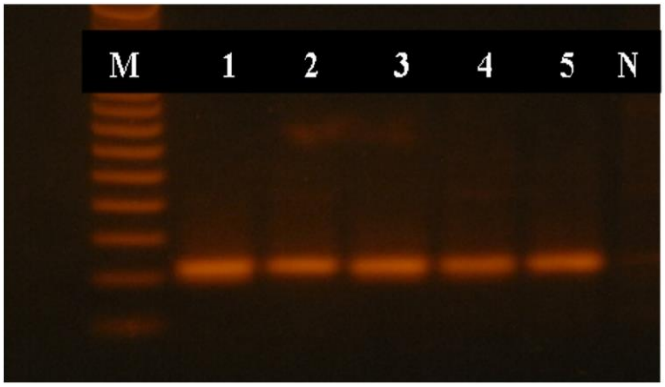

Figure 1. Agarose gel electrophoresis of PCR products of *H. pylori* 16S rRNA positive gene (110 bp) from gastric biopsy on agarose gel. Lane M: molecular weight marker (ladder 50 bp). Lane (1-5): Positive cases of *H. pylori* possessing 16S rRNA gene. Lane N: negative control.

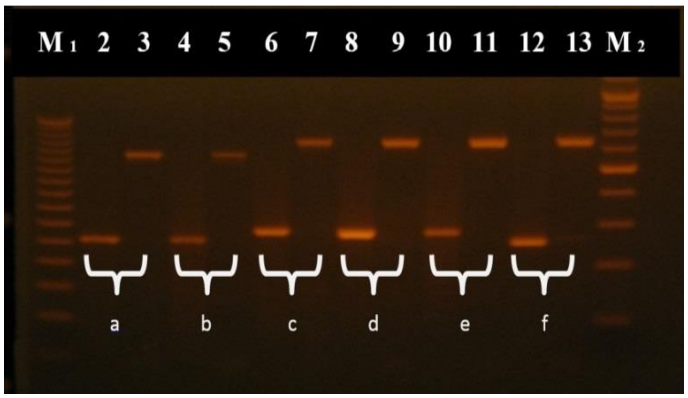

Figure 2. Agarose gel electrophoresis of PCR products of *H. pylori vacA* gene alleles from gastric biopsy, *s1*:259 bp, *s2*:286 bp, *m1*:570 bp, *m2*:642 bp. Lane M1: molecular weight marker (ladder 50 bp). Lane (2, 3) and (4, 5): Genotype of samples a and b which was *(s1/m1)*, Lane (6, 7), (8, 9) and (10, 11): Genotype of samples c, d and e which was *(s2/m2)*, Lane (12, 13): Genotype of sample f which was *(s1/m2)*. Lane M2: molecular weight marker (ladder 100 bp).

0.01). Normal gastric mucosa was found in one *H. pylori*-infected patient of *vacA s2/m1* genotype (Table 3).

Relationship of *vacA* genotypes to *cagA* status and clinical outcomes

The *cagA* gene was detected in 38% (15/40) of the studied *vacA* genotypes strains (Figure 3); 60% of which were significantly associated with *vacA s1/m1* genotype (P=0.041) with the development of severe gastritis reaching up to gastric ulcer, 33.3% (5/15) of *cagA*-positive strains were associated with *vacA s1*/*m2* genotype and only one strain was of *s2*/*m2* genotype.

Table 2. Prevalence of *vacA* genotype alleles among the studied 40 *H. pylori* strains.

***vacA* Genotype Alleles(N=40)**	
Genotype alleles	**N (%)**
vacA	
s1	18 (45%)
s2	22 (55%)
vacA	
m1	12 (30%)
m2	28 (70%)

Analysis of TEM examination

Electron microscopy revealed *H. pylori* in its spiral and coccoid forms (Figure 4), as well as cytoplasmic vacuolar degeneration played by the vacuolating toxin (Figure 5). Ultrastructural examination of the tiny gastric biopsies revealed tissue debris of exfoliating degenerated mucosal epithelium with exposure of blood vessels of the lamina propria in many examined ultrathin sections with large lipid accumulation. Inflammatory lymphocytic cells was evident in the examined sections. Curved *H. pylori* and cluster of unidentified bacilli nearby degenerated mucosal gastric structures were depicted in *H. pylori* positive cases. Also, gastric mucous cells with their characteristic granules resting on intact lamina propria were the main cells seen in most of the examined sections. Many of them showed vesiculated endoplasmic reticulum and vacuolated cytoplasm.

DISCUSSION

Little is known about the geographic distribution of *H. pylori* genotypes in distinct regions, particularly in Egypt. The *vacA* genotypes and its relationship to clinical outcomes, *cagA* status and TEM findings were investigated in *H. pylori* strains from 40 infected Egyptian patients possessing *vacA* gene.

Different results have been reported in studies related to *vacA* s and *H. pylori* strains. In the current study, the *vacA s2* allele was the most predominant genotype (55%) followed by *vacA s1* (45%). Our findings are similar to an earlier report from Egypt (Van Doon et al., 1999), Korea (Kim et al., 2001) and Turkey (Ozbey and Aygun, 2012). Al Quabandi et al. (2005) reported that North Africans

Table 3. Relationship between *H. pylori vacA* genotypes and clinical outcomes in 40 *H. pylori* infected patients.

Endoscopic findings	*vacA* genotypes			
	s1/m1 (n= 11)	***s1/m2 (n= 7)***	***s2/m2 (n= 21)***	***s2/m1 (n= 1)***
Gastritis (n= 23)	5 (21.7)	3 (13.0)	15 (65.2) **	0 (0.0)
Peptic ulcer (n= 7)	4 (57.1) **	1 (14.3)	2 (28.6)	0(0.0)
Normal gastric mucosa (n= 1)	0 (0.0)	0 (0.0)	0 (0.0)	1 (100)
Others[#] (n=9)	2 (22.2)	3 (33.3)	4 (44.4)	0 (0)

Data were expressed as number (%). [#] Other endoscopic findings which include; oesophageal varices, gastro-oesophageal reflux, gastric prolapse **P< 0.01= highly significant.

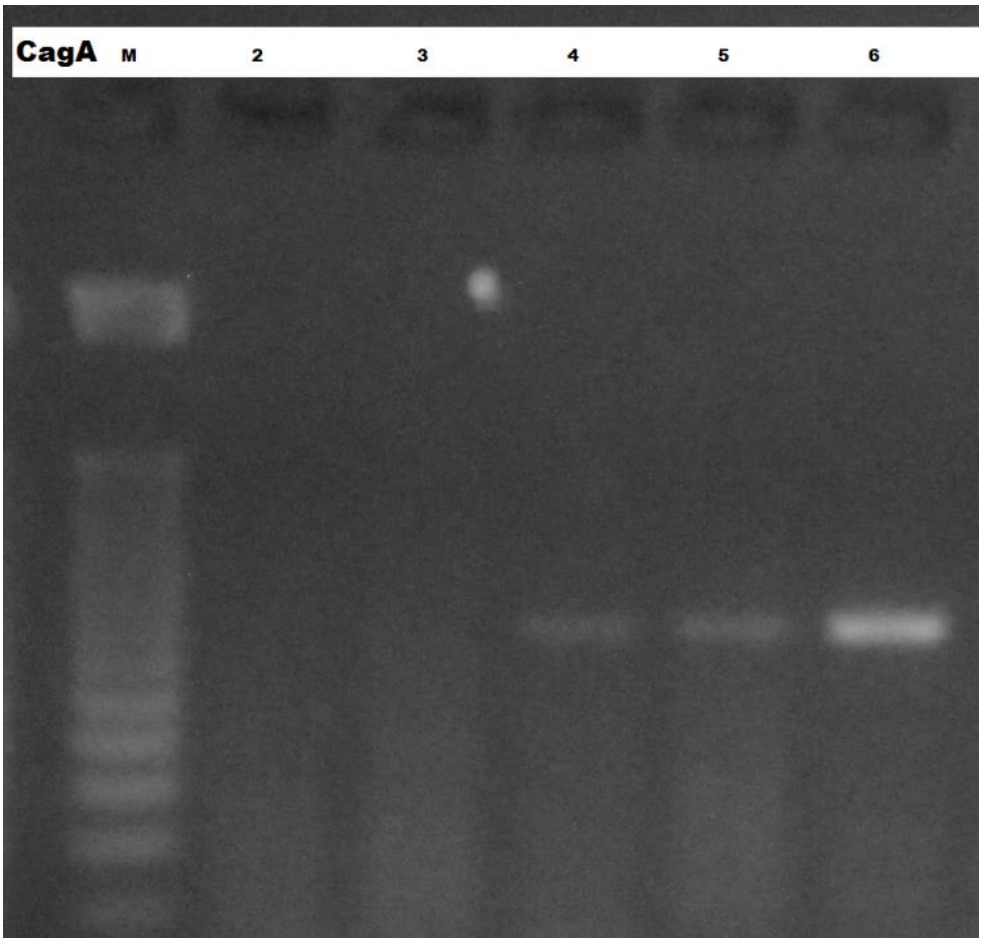

Figure 3. Agarose gel electrophoresis for PCR products of *H. pylori* of *cagA* status. Lane M: Molecular weight marker (50 bp). Lane 2: Negative control. Lanes 4-6: Positive *cagA* gene (400 bp).

were predominantly infected with the *s2* type. In contrary to our results, the previous study from Kuwait reported that *vacA* s1 and s2 types were detected in approximately equal numbers in biopsies obtained from patients of Middle Eastern origin. Previous reports from Cyprus (Krashias et al., 2013) and Saudi Arabia (Sugimoto et al., 2009) revealed that *s2* is the main allele among their *H. pylori* strains.

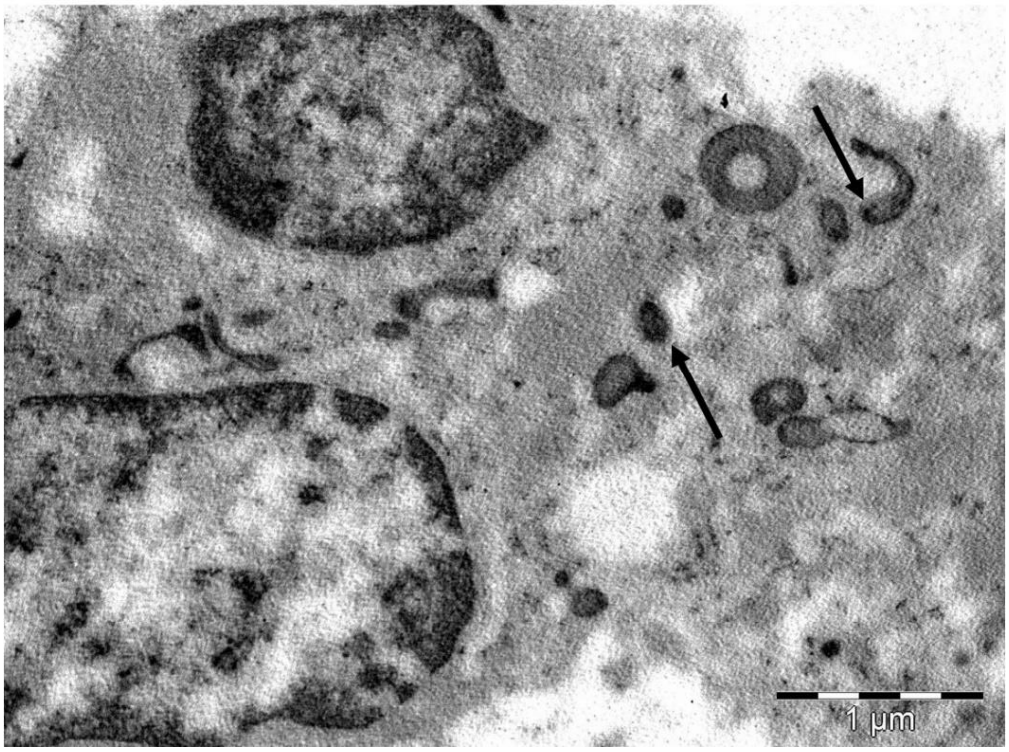

Figure 4. Electron micrograph of gastric biopsy showing spiral (arrow) and coccoid (arrow) forms of *H. pylori.*

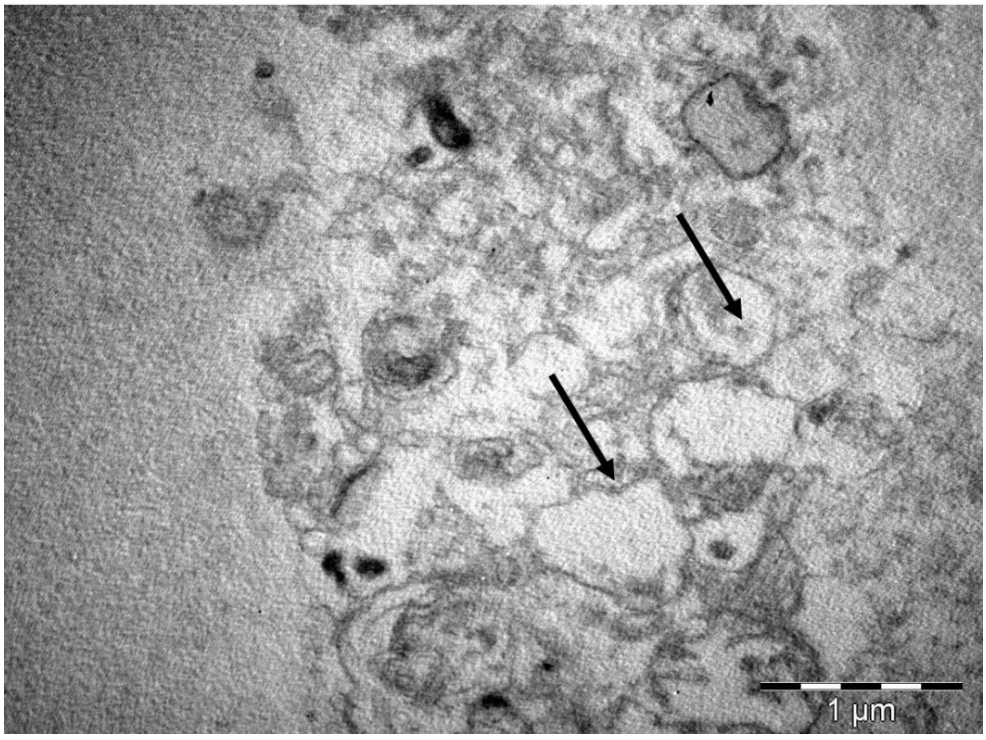

Figure 5. Electron micrograph of *H. pylori* infected gastric mucosa showing cytoplasmic vacuolar degeneration (arrow) caused by the vacuolating toxin.

The m-region encodes the *vacA* binding site to host cells, which appears to be more effective in the *m1* than in *m2* forms (Ferreira et al., 2014). Similar to previous studies in Turkey, Iraq, Iran, Saudi Arabia (Hussein, 2010) and China (Chung et al., 2010), *m2* was predominantly found in all studied *H. pylori* strains (70%) previously reported. However, this was inconsistent with previous results obtained from Egypt where equal distribution of *vacA m1* and *m2* was found (Amer et al., 2013). Genetic variation within virulence factors may account for differences in the pathogenic properties of strains, and thus may help to explain the discrepancies between the number of infected individuals and those that end up developing gastric cancer (Ferreira et al., 2014).

The allele combination between *vacA s* and *vacA m* genotypes and their association to gastroduodenal disorders differ greatly (Nimri et al., 2006). In this study, the prevalence of the *vacA* genotypes *s1m1* was 27.5%, and 57.1% of which was highly significant in relation to peptic ulcer. This is in agreement with previous studies in which association between this genotype and severe gastric outcomes was recognized (Marie, 2012). It is worth to mention that, in these cases long curved virulent spiral forms of *H. pylori* were detected at the level of electron microscopy in association with ulcerated and degenerated mucosal lining. Also, multiple bacterial microorganisms exhibiting curved appearance were seen among the mononuclear inflammatory cells undergoing apoptotic changes and in between the debris of degenerated mucosal cells. It was reported that the long spiral form facilitate the penetration and movement of the microorganism through the mucous gel. *H*. *pylori* lacking the spiral form loses its infectiousness (Bai et al., 2010). Moreover, in this study cytoplasmic vacuolar degeneration of mucosal cells with swollen mitochondria and dilated rough endoplasmic reticulum denoting the presence of the *vacA* gene toxic effect was an important finding. *(*Atherton et al., 1995) and Bai et al. (2010) reported that the pivot role in cell damage induced by *H. pylori* is played by vacuolating toxin.

The significant association between *vacA s1/m1* genotypes and gastric carcinoma development has been substantiated by meta- analysis using reports of patients from diverse geographic origin, including Western, Middle East, Latin America and Africa countries. In an observational longitudinal study from Spain, the progression of premalignant lesions was more frequent in patients infected with *vacA s1/m1* strains than those infected with less virulent *vacA s2/m2* strains (Ferreira et al., 2014). Our data emphasizes the significant association of the most predominant (52.5%) allele combination *s2/m2* with gastritis (65.2%). This predominance goes in accordance with Benenson et al. (2002) and El-Gharbawy et al. (2006). This similarity in *H. pylori* genotypes in three neighboring countries, Egypt, Jordan, and Gaza strip, indicates a geographic influence, which was reported by Abu Amra (2010). Whereas in Egypt, Amer et al. (2013), reported that all possible combination of vacA s1 with m were recognized in their work, in addition, *H. pylori* virulence could not be predicted in relation to different genotypes.

The prevalence of *cagA* (38%) in this study was in agreement to previous report from Egypt (El-Garbawy et al., 2006) and lower than recent studies from Turkey (71.4%) (Ozbey and Aygun, 2012), Korea (97%) (Kim et al., 2001), Malaysia (94%) (Ramelah et al., 2005) China (96.3%) (Zhou et al., 2004) and Kuwait (53%) (Al Quabadi et al., 2005).

Regarding the relationship between *H. pylori* genotypes and clinical outcome, we found a significant association between *cagA* status and *vacA* s1/m1 genotype with development of severe gastritis reaching up to gastric ulcer. These findings are in agreement with Marie (2012) and Ozbey and Aygun (2012).

The attribution between the presence of *cagA* and severe clinical outcome has been a controversial point; as it was previously found that colonization with cagA positive strains has been associated with a fivefold increased risk for diagnosis of duodenal or gastric cancer (Diab et al., 2009). Whereas in other studies the occurrence of gastric malignancy was independent of *cagA* status and others implicated the pivotal roles of other virulence factors (*cag E, cag T*, *vac A* and *bab A*) in the aetiology of gastric cancer (Marie, 2012).

Conclusion

In Egypt where prevalence of *H. pylori* infection is high, genotyping of *H. pylori* virulence factors can help to predict patients who are at a high risk of related gastroduodenal diseases. Although *H. pylori* with *vacA s2*/*m2* genotype is mostly related to low level of virulent strains yet, significant crosstalk between *H. pylori* strains harboring both *vacA s1*/*m1* and *cagA* gene provides crucial insights into virulence of high level.

Conflict of Interests

The authors have not declared any conflict of interests.

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