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Molecular detection of protein S and M subunits of *Helicobacter pylori* vaca toxin in some Egyptian patients

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Helicobacter pylori associated with several gastrointestinal manifestations in a substantial population all over the world. The *vacA* gene which encodes 88 kDa vacuolating toxin (VacA) is an important virulence determinant. The variation in S and M alleles of the *vacA* gene affects the amount of toxins produced by the organism and hence its virulence. The study was aiming to determine the spread of the organism and the distribution of the *vacA* gene different alleles in Egyptian population and hence identify the risk of complications in these patients. Thirty-five biopsy specimens were taken from cases attended the Gastroenterology endoscopy Unit at Zagazig university hospital for various indications and a written consent was taken. The specimens were investigated for *Helicobacter pylori* infection using urease test, culture and molecular techniques. The genotypes of the *vacA* gene alleles were identified by PCR. en samples were proven to be positive for the microbe and confirmed using biochemical reaction and PCR for the 16S rRNA region. The S1 and S2 alleles were found at (20% and 60%) respectively while in 20% the allele was not detected. The M1 allele was not detected at all while the M2 allele was detected in 50% of isolates and 50% were not identified. Two samples were S1M2, three samples were S2M2, and three samples were S2 while the M allele was not identified in them. In one/Two sample/s neither of both alleles were identified.

Keywords: ***Helicobacter pylori*, *vacA* gene, S and Protein, Antibiotic resistance**

INTRODUCTION

Helicobacter pylori is a microaerophilic spiral-shaped bacterium that resides in the mucous layer of the human stomach. The organism can establish itself without symptoms in the majority of carriers for years or decades (Blaser 1990). However, in a small proportion of hosts, the organism may induce numerous gastrointestinal manifestations such as gastritis, peptic ulcer and gastric carcinoma (GC) (Hopkins 1994). The clinical outcome of the infection depends on host immunologic defense mechanisms, environmental factors, and/or the virulence capacity of the bacteria (Akopyanz 1992).

Several genes, including *cagA* and *vacA*, have been identified that likely play a role in the pathogenesis of *H. pylori* infection. The *cagA* gene which encodes the CagA protein is present in more than 50% of *H. pylori* strains (Covacci, 1993). It is a constituent of a pathogenicity island (*cag*PAI); a 40-kbp DNA insertion containing genes that encode a type IV

secretion system (Censini 1996), and it is said to be associated with gastric mucosal atrophy, duodenal ulcer, and gastric carcinoma (Nomura 1995).The *vacA* gene is present in all *H. pylori* strains and encodes an 88-kDa vacuolating toxin (VacA) that affects epithelial cells and which is important in the pathogenesis of peptic ulcer and gastric adenocarcinoma (Atherton 1995). Within the *vacA* gene, two regions of marked sequence diversity can be distinguished. The s-region (encoding the signal peptide) is present as either an *s1* or an *s2* allele, while the m-region (the middle region of the toxin) can be either *m1* or *m2* (Van Doorn, 1999). The combination of *s*- and *m*-region alleles determines the produc ability of the vacuolating cytotoxin and is associated with the pathogenicity of the bacterium. VacA *s1*/*m1* strains produce large amounts of vacuolating toxin, *s1*/*m2*strains produce moderate amounts, and *s2*/*m2* strains produce very little or no active toxin (Atherton 1995).The *VacA s2*/*m1* genotype has been reported in Mexican and South African populations (Morales-Espinosa 1999), but those strains are rare. The *VacA s1* and *m1* bearing strains have been associated with increased virulence and greater gastric epithelial damage and ulceration than *s2* and *m2* strains (Atherton 1995).

This work aimed to genotype *H. pylori* strains from gastric biopsies collected from *H. pylori* infected individuals using PCR based technique.

MATERIALS AND METHODS

1-Isolation of samples:

During the period from March 2017 to March 2018, thirty-five biopsy specimens from cases attending endoscopy unit, Zagazig university hospital were collected after a written consent and according to Helsinki declaration proposed ethical statements. The collected specimens were directly transported to the laboratory in 1.5-2 ml BHI broth media (Veenendaal et al., 1993). Each specimen was minced using sterile mortar and pestle and inoculated into BHI agar plates containing Helicobacter pylori selective supplement (SR0147E-Oxoid).The plates were incubated for 3-7 days at 37°c in anaerobic jar under microaerophilic conditions 5% O_2, 10% CO_2 and 85% N_2 (Blaser 1980) using Campy-Gen (Oxoid, UK). Cultures were then inspected for characteristic growth of the microbe.

A rapid urease test was done for each specimen using (>>>) for preliminary diagnosis of H. pylori positive samples.

2-PCR technique:

Total DNA from each specimen was extracted using Qiamp DNA minikit (Qiagen, Germany) following manufacturer protocol for purification from tissues. Three simplex PCR programs was performed for isolated amplification of VacA-s, VacA-m and16srRNA. The three sets of primers used are shown in table 1. The amplified VacAs region resulted in generation of 259bp fragment in case of type s1 variants and a fragment of 286 bp for type s2 variants.

For typing of the VacA gene m region, a universal reverse primer and a single of four forward primers were used separately. The amplification of fragments of 107bp for m1 or182 for m2 strains was inspected for genotyping. Specific 16S rRNA gene primers for H. pylori were used as a positive internal control for each specimen yielding amplimers of 109bp. All PCR mixtures consisted of 10 mmol/L Tris-HCl (pH 8.3), mol/L of each 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 300 µ dNTP, 0.6 µmol of each forward and reverse primer, and 0.6 U of HotStarTaq DNA Polymerase (Qiagen) in a final volume of 20 µL. Five microliters of DNA extracted from gastric biopsies was added to each reaction mixture.

Table 1: PCR primers used in the study

Amplified gene and region	Primer designation	Primer sequence(5"-3")
VacA S1;S2	VA1F VA1R	ATG GAA ATA CAA CAA ACA CAC CTG CTT GAA TGC GCC AAA C
VacA m1;m2	MF1.1F	GTG GAT GCC CAT ACG GCT AA
	MF 1.2F	GTG GAT GCT CAT ACA GCT {AT}A
	MF1.3F	GTG GAT GCC CAT ACG ATC AA
	MF1.4F	GCG AGC GCT CAT ACG GTC AA
	MR1-R	[AG]TG AGC TTG TTG ATA TTG AC
16SrRNA	HP1 HP2	CTG GAG AGA CTA AGC CCT CC ATT ACT GAC GCT GAT TGT GC

The PCR programs for *vacA m* amplification comprised 10 minutes at 95 °C, followed by 60 cycles of 30 seconds at 94 °C, 30 seconds at 48 °C, 30 seconds at 72 °C, and a final extension at 72 °C for 5 minutes. The amplification programs for *vacA s* and 16S rRNA consisted of 10 minutes at 95 °C, followed by 60 cycles of 30 seconds at 94 °C, 30 seconds at 50 °C, 30 seconds at 72 °C, and a final extension at 72 °C for 5 minutes. In all cases, the amplified products were electrophoresed in 2% agarose gels stained with ethidium bromide for 40 minutes at 120 Volts. Positive and negative controls were included in each experiment.

RESULTS

To isolate *H. pylori*, minced biopsy specimens were streaked on BHIA plates with *H. pylori* selective supplement. Results in Table 2 revealed that 8 isolates were grown on the above media after incubation under microaerophilic conditions. These isolates were tested for oxidase, catalase, and urease activities. Only 7 isolates were positive for these enzymes while one isolate (n.13) was catalase negative .Five of these isolates (n.8,9,11,15 and16) were recovered from biopsy of female patients while ,two isolates were recovered from male patients (n.1 and 19). These results are shown in table 2.

POLYMERASE CHAIN REACTION (PCR) RESULTS

DNA from gastric biopsies specimens from 35 adults was amplified using primers specific for s and m alleles of vacA, and for *H.pylori* 16srRNA.

Detection of *H. pylori* Infection by PCR of 35 DNA samples isolated from gastric biopsies, 10 were positive for *H. pylori* (27%) as determined 7 of samples by cultivated on BHI media and tested by some biochemical tests (table 2), the last 3 samples were detected only by rapid urease test (table 3).All the 10 positive samples were tested by PCR for the 16S rRNA region s and m alleles of *H. pylori*.

Among the 35 collected specimens, *H. pylori* Infection was identified in 10 (27%) samples; 7 samples were confirmed by culture on BHI media and biochemical reactions (table 2), and 3 samples were detected only by rapid urease test (table 3). All the preliminary 10 positive samples were tested by PCR for the 16S rRNA region s and m alleles of *H. pylori*.

Detection of 16srRNA Allele of vacA:

All the 10 positive samples were tested by PCR for the 16S rRNA region of *H. pylori*, resulting in: 9(90%) positive and one negative for the 16srRNA region of H. pylori figure1and figure2 .

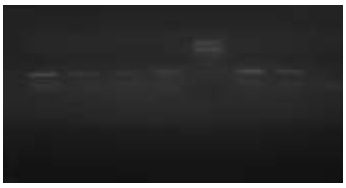

Figure1;16srRNA for the seven cultivated biopsy samples

Figure2: 16srRNA for the three uncultivated biopsy samples

In the9 of those positive

10 samples of H.pylori16srRNA and one was used as negative control all were tested for, s subunits of *vacA* gene by PCR , resulting in:8(88%) out of the 9 were positive and one was not detected(false negative)figure 3.

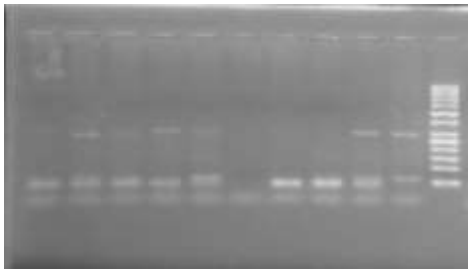

Figure3: S allele for 9positive 16srRNA biopsy samples

Detection of m Allele of vacA:

In the9 of those positive 10 samples of H.pylori16srRNA and one was used as negative control, all were tested for, m subunits of *vacA* gene by PCR, resulting in:5 samples were positive for m2 (55.5%),one had nonspecific band(11.1%) and 2were false negative(22.2%) figure4.

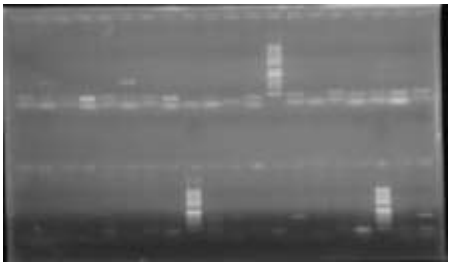

Figure4.mallel for the 10 positive H. pylori{one negative for 16srRNA(negative control),and one negative for s allel(false negative),eight samples were be tested for m allele}.

Detection of s and m Alleles of vacA Genotyping:

Amplification of *vacA s* and *m* alleles allowed concomitant discrimination of *s1* and *s2* alleles as signals of 259 bp and 286 bp, respectively, and of *m1* and *m2* alleles as 107 bp and 182 bp fragments, respectively. Genotyping for the *vacA m* fragment using a set of primers for simplex PCR allowed detection of 5 *m2*signals only but no m1were be detected from8 positive samples for s allele.

Genotyping for the *vacA s* fragment using a set of primers allowed detection of 6 bands ofs2,and 2 bands of s1 from8 positive samples for s allele.

The central finding of this study, resulting from *H. pylori* genotype analyses from biopsies, is that the high incidence of GC is associated with an increased incidence of specific *H. pylori* genotypes. These results are in line with (Gong et al., 2005),the more virulent *s1m1* strains the more probably of GC as*vacA s1*/*m1* strains produce large amounts of vacuolating toxin, *s1*/*m2*strains produce moderate amounts, and *s2*/*m2* strains produce very little or no active toxin .These results are in line with.(Atherton JC et al., 1995) .In this studyin Egypt the allele m1 was not found but only m2.Of the 9positive16srRNA two samples were s1m2,three samples were s2m2 ,one sample was false negative ,and the other three weres2 but were not detected for m allele .These results are in line with.(Yamaoka Yet,al.2002).

Table 2: Microaerophilic growth and some biochemical identification tests for 25 collected biopsy specimens:

Sample number	Gender		Age	Growth	Gram stain	Biochemical tests			
	Male	Female				Oxidase	Catalase	Urease	Rapid urease
1	•		37	+	•	+	+	+	+
2	•		43	-	ND	ND	ND	ND	ND
3		•	55	-	ND	ND	ND	ND	ND
4	•		40	-	ND	ND	ND	ND	ND
5		•	32	-	ND	ND	ND	ND	ND
6	•		21	-	ND	ND	ND	ND	ND
7	•		26	-	ND	ND	ND	ND	ND
8		•	35	-	ND	ND	ND	ND	ND
9		•	41	+	•	+	+	+	+
10		•	38	-	•	+	+	+	+
11		•	48	+	•	+	+	+	+
12		•	22	-	ND	ND	ND	ND	ND
*13	•		34	+	•	+	ND	ND	ND
14	•		50	-	ND	ND	ND	ND	ND
15		•	27	+	•	+	+	+	+
16		•	41	+	•	+	+	+	+
17		•	43	-	ND	ND	ND	ND	ND
18	•		34	-	ND	ND	ND	ND	ND
19	•		20	+	•	+	+	+	+
20	•		39	-	ND	ND	ND	ND	ND
21		•	42	-	ND	ND	ND	ND	ND
22		•	28	-	ND	ND	ND	ND	ND
23	•		44	-	ND	ND	ND	ND	ND
24	•		26	-	ND	ND	ND	ND	ND
25		•	40	-	ND	ND	ND	ND	ND

•Gender of patient specimen,+ positive for growth, and tested enzymes,-Negative for growth and tested enzymes, 13 *this sample was negative for catalase and urease and was neglected through study, ND not detected.

Table 3:-Rapid urease test and PCR tests for 10 collected biopsy specimens:

Biopsy number	Gender		Rapid urease test	PCR 16S rRNA	PCR for S subunit of vacA gene	PCR for m subunit of vacA gene
	Female	Male				
1	•		ND	ND	ND	ND
***2**	•		**+**	ND	ND	ND
3	•		ND	ND	ND	ND
4		•	ND	ND	ND	ND
5		•	ND	ND	ND	ND
6	•		ND	ND	ND	ND
7		•	ND	ND	ND	ND
8	•		**+**	**+**	**+**	**+**
9	•		**+**	**+**	**+**	**+**
10		•	ND	ND	ND	ND

Gender of patient specimen,+ positive for Rapid urease test , and PCR tests,*2this sample was negative for16srRNA, s and m subunits and was neglected throughout study, ND not detected.

Table4: PCR results tests for the 25 specimens biopsy.

Sample number	Detection of S and M subunits of VacA toxin by PCR on biopsy specimens		
	16SrRNA	S subunit of vacA gene	M subunit of vacA gene
1	+	+	+
2	ND	ND	ND
3	ND	ND	ND
4	ND	ND	ND
5	ND	ND	ND
6	ND	ND	ND
7	ND	ND	ND
8	+	+	+
9	+	+	+
10	+	+	ND
11	+	+	ND
12	ND	ND	ND
13	ND	ND	ND
14	ND	ND	ND
*15	+	ND	ND
16	+	+	+
17	ND	ND	ND
18	ND	ND	ND
19	+	+	ND
20	ND	ND	ND
21	ND	ND	ND
22	ND	ND	ND
23	ND	ND	ND
24	ND	ND	ND
25	ND	ND	ND

positive for PCR tests,*15this sample was negative for s and m subunits PCR tests and was neglected through study, ND not detected.

Table 6: Number and percentage of the10 positive biopsy specimens:

Number of positive16srRNA Of the 10 positive H.pylori	%	Number of positive s allele		%	Number of positive m allele		%
9	90%	S1	S2		M1	M 2	
		2	6	88%	non	5	55,5%

CONCLUSION

The distribution of *vacA* gene alleles in *H. pylori* isolated from Egyptian population and risk of complications in patients was assessed. Thirty-five biopsy specimens were taken from cases attended the Gastroenterology endoscopy Unit at Zagazig university hospital. The S1 and S2 alleles were found at 20 and 60%, respectively. The M1 allele was not detected at all, while the M2 allele was detected **in 50% of isolates and 50% were not identified.**

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

RAH and EYE designed and performleed the experiments, MAY, OMB wrote and edit the manuscript, RSE performed the experiments. All authors read and approved the final version.

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