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ISOLATION OF CagA AND VacA GENES FROM *H. PYLORI* INFECTED PATIENTS WITH VARIOUS GASTRODUODENAL LESIONS

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Abstract

This study aimed to learn the incidence of *Helicobacter pylori* infection in patients with various gastroduodenal endoscopic lesions and the frequency of virulence *H.pylori* associated genes CagA and VacA in these patients.

One hundred seventy six patients (96 males and 80 females) attending endoscopy units for various dyspeptic symptoms were studied.

Antral biopsies were obtained to detect *H.pylori* by rapid urease test, culturing and histopathologic examination. Twenty five patients with positive *H.pylori* isolates who were found to be mannose resistant, were tested for cytotoxic associated (CagA) and vacuolating cytotoxin A (VacA) genes.

Among studied patients, positive *H.pylori* detected by rapid urease test, culturing and histopathologic examination (from 50 patients only) were 113 (63%), 127 (71%) and 25 (50%) respectively.

Out of 25 patients with positive *H.pylori* isolates who were found to be mannose resistant, positive genes of either CagA or VacA were detected in 18 (72%) patients with positive isolates, while positivity of both genes were detected in 13(52%) patients with positive isolates. Five (45.4%) and 5 (45.4%) out of patients with duodenal ulcers and gastritis respectively were positive for both (CagA) and (VacA) genes.

In conclusion, the highest detection rate of *H.pylori* infection was by bacterial culture. A correlation between CagA and VacA genes and endoscopic lesions of duodenal ulcers and gastritis was found.

Introduction

H.pylori is a human specific pathogen lives deep beneath the mucus layer closely attached to the gastric epithelium[1]. The infection with *H. pylori* is the most frequent infection worldwide as more than half of the world's population are infected[2].

H. pylori is now recognized as the cause of gastritis and most cases of peptic ulcer disease. Its long term carriage increases the risk of gastric adenocarcinoma six times and it is designated as a class I carcinogen[3]. *H. pylori* has also been implicated as a cause of gastric mucosa associated lymphoid tissue (MALT) lymphomas.[4]

It is estimated that 0.4–1.0% of uninfected adults acquire *H. pylori* each year, and the incidence of *H. pylori* infection tends to increase with age[5]. However, detailed information of the prevalence of the bacteria in developing countries and on the factors that may influence the pattern of distribution remains scanty. The main risk factors for *H. pylori* acquisition are childhood, low

socio-economic status, the presence of *H. pylori* positive family members, and poor sanitation[6,7].

The mechanism by which *H. pylori* causes gastric mucosal damage and peptic ulcer is not fully elucidated, but it is clear that it involves several *H. pylori* virulence factors enabling it to survive within the strong gastric acid. In addition, host dependent noxious substances lead to progressive damage to the gastric mucosa, deregulation of acid production, induction of gastric metaplasia and development of chronic gastritis. These *H. pylori* virulence factors and host noxious substances include cytotoxin associated gene A (CagA), vacuolating cytotoxin A (VacA), ammonia, lipopolysaccharide (endotoxin), platelet activating factors, nitric oxide and interleukin–8 [8,9].

Vac A cytotoxin, which is synthesized as a 140KDa precursor and released from the bacterium as a 95KDa mature toxin[10]. Vac A cytotoxin secreted by about 50% of *H. pylori* isolates in western countries[11].

Infection with vacuolating cytotoxin positive strains is reported to be associated with peptic ulcer disease. They showed that strains with vacuolating cytotoxin activity were found in 16 out of 24(67%) patients with peptic ulcers but in only 16 of 53 (30%) patients without[12-15].

Infection with *H. pylori* strain expressing cytotoxin associated Cag A protein is more virulent and is associated with increased risk of development gastric cancer[16]. There is evidence that Cag A positive strains are more infectious [17], achieve higher bacterial density on the gastric mucosa and cause more inflammation than Cag A negative strains. Cag A positive *H. pylori* strains have been shown to be associated with interleukin–8 (IL.8) induction in gastric epithelium, neutrophilic infiltration and thus be related to gastric inflammation and gastroduoderal diseases[18,19].

Expression of Cag A protein is closely associated with that of vacuolating cytotoxin[20], although the underlying mechanism is not understood,thus Xiang *et al.*[21] classified *H. pylori* strains in two groups type I and type II. These studies stated that type I strains, which are positive for both Vac A and CagA, were strongly associated with peptic ulcer diseases in the host[21,22].

The present study was conducted with the following objectives; To show the incidence of *Helicobacter Pylori* in patients with variable gastroduodenal endoscopic lesions. To study the frequency of virulence associated genes Cag A and Vac A in the studied patients with positive *H. pylori* isolates.

Patients and methods

A total of 176 patients 96 males and 80 females, whose ages ranged from 14 – 80 years, with various dyspeptic symptoms attending endoscopy unit at Al-sadder Teaching Hospital (Basrah) during the period from October 2004 through August 2005 were underwent upper gastroduodenal endoscopic examination. Patients with recent upper gastrointestinal bleeding, on non-steroidal anti inflammatory drugs, antibiotics or proton pump inhibitors were excluded from the study.

Two antral biopsy samples were obtained from each patient one for rapid urease test and the second for bacterial culturing as described by Barn *et a*[23]. A third biopsy sample was obtained from (50 patients only) due to cost constraints for histologic examination. Culture colonies that exhibited characteristic morphologies were studied also biochemically by catalase and oxidase according to Finegold and Baron[24] to confirm the identity of positive *H. pylori* isolates. Patients from whom *H. pylori* were isolated and identified biochemically were studied for toxigenic strains by mannose resistant heamagglutination test (MRHA)[25].

Grown cultures were suspended in phosphate buffer saline (PBS) at pH. 7.2[26], and tested against human erythrocyte. Blood was drawn from volunteer donors and placed into a tube containing 1.0 ml of 3.8% citric acid, in distilled water per 9.0 ml of blood. Blood was diluted 1:4 with PBS to test for mannose resistant haemagglutination. MRHA test was performed by mixing equal volume of each bacterial suspension with erythrocyte suspension, with and without (1%) D-mannose, in PBS. Glass tile with round depressions were rocked gently with swirling action at (20–22C). Mixture was subsequently incubated at (4° C)for up to (20 min) before reading. Positive toxigenic strains, which revealed haemagglutination with erythrocyte cells, were preceded by polymerase chain reaction (PCR) technique by using primer sequences. table I[27].

Table I:Oligonucleotide primer sequences of Cag A and Vac A for *H. pylori* clinical isolates

Primers	Primer sequences		Lenth	Tm	TA
CagA gene	Forward	5- AGTAAGGAGAAACAATGA-3	١٨	48C	56C
	Reverse	5-AATAAGCCTTAGAGTCTTTTTGGAAATC-3	28	74C	٥٦C

Primers	Primer sequences		Lenth	Tm	TA
VacA gene	Forward	5-GCTTCTCTTACCACCAATGC-3	٢٠	60C	55C
	Reverse	5-TGTCAGGGTTGTTCACCATG-3	20	60C	٥٥C

***Tm= melting temperature, TA= Annealing temperature**

Results

Sex and age distribution of patients are illustrated in table II. Ninety-six (54%) and 80(45.5%) were males and females respectively. Forty-nine (52%) of males and 46 (57.5%) of females showed positive *H. pylori* isolates by culture, the differences were statistically not significant (P>0.05).

Table II: Age and sex distribution of patients with *H. pylori* infection.

Sex		Age groups (years)						
		≤20	21–30	31–40	41–50	51–60	> 60	Total
Male	+/total	0/2	9/18	15/31	7/11	7/14	11/20	49/96
	%	0%	50%	48.4%	63.6%	50%	55%	52%
Female	+/total	2/5	8/14	5/11	10/19	10/16	11/15	46/80
	%	40%	57.1%	45.6%	52.6%	62.5%	73.3%	57.5%
Total	+/total	2/7	17/32	20/42	17/30	17/30	22/35	
	%	28.5%	53.1%	47.6%	56.7%	56.7%	62.8%	

The highest detection of *H. pylori* isolates was recorded in the age group more than 60 years 22/35 (62.8%), while the lowest detection rate was recorded in the age group below 20 years 2/7 (28.5%), the differences were statistically not significant (p>0.05).

Out of 176 studied patients ,124 (70.4%) showed abnormal endoscopic findings. In 52 (29.5%) patients, the endoscopic examination was normal table III.

Table III: Endoscopic findings, Rapid urease test&culture results in studied cases.

Endoscopic findings	Total No.	No. and (%) of RUT +ve patients	No. and (%) of Culture +ve patients
Ca. Stomach	10	6 (60)	10(100)
Gastric ulcer	19	13(69)	15(79)
Duodenal ulcer	12	8 (67)	9(75)
Gastric and duodenal ulcer	1	1(100)	1(100)
Gastritis and gastric ulcer	4	2(50)	4(100)
Gastritis	62	40(64.5)	43(70)
Gastritis and duodenitis	5	3(60)	5(100)
Gastritis and duodenal ulcer	6	4(67)	5(83.3)
Duodenitis	5	4(80)	5(100)
Total	124	81(64)	97(77)
Normal	52	32(61.5)	30(57.6)
Total	176	113(64.2%)	127(72.2%)

Table III also shows positive *H. pylori* cases detected by (RUT) and culture. RUT and culture were positive in 81 (64%) and 97 (77%) out of 124 with positive endoscopic lesions respectively, while RUT and culture were positive in 32 (61.5%) and 30 (57.6%) out of 52 with normal endoscopic examination.

Table IV shows the histopathologic findings and positive *H. pylori* among 50 of examined biopsy specimens, 24 (48%) of biopsy specimens showed abnormal histopathological findings. *H. pylori* was positive in 3 (75%) 6 (54.5%) and 3 (33.3%) of histopathologic specimens with severe, moderate and mild gastritis respectively, 26 (52%) of biopsy specimens showed normal gastric histology and all were negative for *H. pylori.*

Table IV: Histopathological findings and positive *H. pylori* among the examined 50 biopsies.

Histopathological findings	No. (%)	*H. pylori* seen	
		No.	(%)
Severe gastritis	4 (16.6)	3	75
Moderate gastritis	11 (45.8)	6	54.5
Mild gastritis	9 (37.5)	3	33.3
Total	24 (48)	12	50
Normal gastric tissue	26 (52)	0	0

Twenty five patients with both positive *H. pylori* isolates and endoscopic findings who were found to be mannose resistant, were tested for the presence or absence of cytotoxic associated A (CagA) and vacuolating cytotoxic A (VacA) genes, both representing toxigenic strain by using polymerase chain reaction (PCR) techniques (table v). Out of 25 patients, positive genes of either CagA or VacA were detected in 18 (72%) patients with positive isolates, while positivity of both genes were detected in 13 (52%) patients with positive isolates.2 (8%) did not yield any PCR product of both of the above genes. The statistical difference was significant ($p<0.05$) between Cag A positive and negative,Vac A positive and negative and both Cag A positive and negative.(table VI).

Table V: PCR detection of selected genes (CagA and VacA) in patients with *H. pylori* isolates

Strain	Endoscopic diagnoses	Cag A	Vac A	Strain	Endoscopic diagnoses	Cag A	VacA
28	Gastric ulcer	–	–	153	Duodenal ulcer	–	+
56	Duodenal ulcer	+	–	155	Ca. stomach	+	+
59	Gastritis	–	+	160	Gastric ulcer	+	–
61	Gastritis	–	+	161	Gastritis	+	+
62	Duodenal ulcer	+	+	163	Gastritis	–	+
65	Duodenal ulcer	+	+	165	Gastritis	+	–
68	Duodenal ulcer	–	+	169	Duodenal ulcer	+	+
76	Duodenal ulcer	+	+	170	Gastric ulcer	+	+
86	Duodenal ulcer	+	–	171	Gastric ulcer + Duodenal ulcer	+	+
89	Gastritis	–	–	174	Gastritis	+	+
98	Gastritis	+	+	179	Gastritis	+	+
105	Gastritis	+	–				
108	Gastritis	+	+				
144	Duodenal ulcer	+	+				

Table VI: Identification of CagA and VacA genes by PCR in *H. pylori* positive isolates from 25 patients with endoscopic lesions

Endosc. Diagnos. No.(%)	Number &(%) of samples							
	CagA +	CagA -	VacA+	VacA-	CagA+ VacA-	CagA- VacA +	CagA- VacA-	CagA+ VacA+
Duod. Ulcer 9 (36)	7 (77.7)	2 (22.2)	7 (77.7)	2 (22.2)	2 (22.2)	2 (22.2)	0 (0)	5 (55.5)
Gastritis 11 (44)	7 (63.6)	4 (36.3)	8 (72.7)	3 (27.2)	2 (18.1)	3 (27.2)	1 (9)	5 (45.4)
Gastric ulcer 3 (12)	2 (66.6)	1 (33.3)	1 (33.3)	2 (66.6)	1 (33.3)	0 (0)	1 (33.3)	1 (33.3)
Duod. ulcer & gastric ulcer 1 (4)	1 (100)	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	1 (100)
Ca. Stomach 1 (4)	1 (100)	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	1 (100)
Total 25	18 (72)	7 (28)	18 (72)	7 (28)	5 (20)	5 (20)	2 (8)	13 (52)

P < 0.01

Discussion

In the present study, no statistically significant differences (P>0.05) were found in the detection of *H. pylori* among males (52%) and females (57.5%). These differences were also not reported in other studies[28,29].

Regarding the age groups, the highest detection rates of bacteria were recorded in the age group more than 60 year (62.8%), while the lowest detection rates were recorded in the age group below 20 year(28.5%)This result was nearly similar to other study[30]. The higher prevalence of the infection in elderly people is partly due to a cohort effect[31], in addition, the low immunity in the elderly may be a cause of increased incidence in this age group.

In the present study, the percentage of positive biopsy rapid urease test was 64% (81/124) of positive endoscopic lesions ,which was similar to other several studies[30,32-34]. The use of biopsy urease test in the endoscopy unit is convenient since it is easy to perform and read and sensitive enough[35] .

Culture of gastric biopsies is the golden standard technique for *H. pylori* diagnosis, although primary isolation of *H. pylori* from a biopsy specimen is a difficult process, but the typical reports success rates in culturing the organisms are in the range of 70% - 80% to 95% sensitivity and 100% specificity[36] In the present study, the total isolation rate of *H. pylori* was reaching a percent of 77% (97/124) of positive endoscopic lesions (table III). This rate is compatible with the above mentioned international results[36]

There are several factors, which might affect culturing of the organism. Patchy distribution and loss of viability of the organism during transportation may be responsible for a poor negative predictive value associated with culture of *H. pylori*. *Helicobacter pylori* provokes an inflammatory response that damages the

gastric protective mucosa and exposes the mucosal surface to acid and pepsin[37-39]. Some investigators have shown that this inflammatory response is associated with the organisms ability to secrete vacuolating cytotoxin or release lipo-polysaccharides, which in turn induce recruitment and release of soluble mediators of inflammations including interleukin 8[28,40]. These mediators alone or in combination induce inflammatory response[41,42]. In this study, inflammatory changes in antral biopsy specimens were seen in some cases. The remaining negative cases may be explained by a patchy distribution of the organism, the density of *H. pylori* which can vary in different sites and inter observer variability[43].

Out of the total isolates, twenty-five *H. pylori* isolates were chosen for the molecular diagnosis by using PCR technique.

This technique regards a modern developed and one of the most accurate techniques in the diagnosis of the *H.pylori* depending on the DNA of the bacterium. Amplification by PCR makes it possible to detect tiny amounts of *H.pylori*'s DNA[44].

Potential benefits also include its high sensitivity and the potential to identify toxigenic strains on the basis of the specific genes that they possess[45].

In the present study PCR technique was used for the detection of virulence associated genes (CagA and VacA genes) in the isolates of *H.pylori* .The study shows that 18 of 25 isolates (72%) were positive for either CagA or VacA genes, 13(52%) were positive for both CagA and VacA genes. These results were in line with the other studies in this field[40]. Atherton *et al*[39] and Wong *et al*[46] had detected Vac A gene in 93% and 95.8% of *H. pylori* positive gastric biopsy specimen by using the PCR in Netherlands and Hong Kong respectively.

Expression of CagA protein is closely associated with that vacuolating cytotoxin[20], although the underlying mechanism is not understood. Maeda *et al*[11] reported that most *H. pylori* strains isolated in Japan were positive for vacuolating cytotoxin and CagA protein.

Fifty-four out of sixty-eight (79%) isolates were positive for the vacuolating and CagA protein and were therefore type I strains. Weel *et al*[22] reported that infection with type I strains was found in 43/76 (57%) patients with peptic ulcers, but in only 28/76 (37%) patients with non-ulcer dyspepsia in the Netherlands.

In conclusion, *H. pylori* has proved to be one of the most genetically divers bacteria yet found. Almost any technique that looks at gene arrangements or that disrupts the chromosome, e.g. digestion by restriction enzymes, shows that strains from each individual are different. Genetic diversity in *Helicobacter pylori* strains may affect the function and antigenicity of virulence factors associated with bacterial infection and, ultimately, disease outcome.

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