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Study of Helicobacter pylori cag A status and Interleukin -18 in patients with dyspepsia

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Abstract:

Helicobacter pylori (H.pylori) is a Gram negative microaerophylic bacterium that is closely associated with gastroduodenal diseases. The prevalence of H.pylori infection is high world wild. Cytotoxin –associated protein (cag A)encoded by cag A gene has been reported to be a major virulence factor of H.pylori infection. The aim of this study is to investigate the H.pylori cag A status and its relationship to endoscopic and histopathological findings as well as serum and local interleukin-18 (IL-18) production. Sera and antral biopsy were taken from 40 patients with dyspepsia.The presence of H.pylori was confirmed by culture, histopathology and polymerase chain reaction (PCR),while cag A gene was detected by PCR. Serum and gastric tissue IL-18 were measured. H.pylori was detected in 30 out of the 40 patients (75%) and cag A gene was detected in 16 out of 40 (40%) and was absent in 14 out of 40 (35%) patients.Ten out of the 40 patients (25%) were H.pylori -negative. Serum Il-18 was significantly higher in cag A-positive patients as compared to cagA –negative H.pylori infected patients ($p<0.001$)and to H.pylori-negative patients ($p<0.001$). Tissue IL-18 also showed significant increase in cag A-positive patients as compared to cag A-negative patients ($p<0.05$) as well as compared to non infected patients ($p<0.001$).There was significant positive correlation between serum and tissue IL-18 ($r=0.951.p<0.01$). Also, there was significant positive correlation between tissue IL-18 and mucosal H.pylori density ($r=0.072;p<0.01$), neutrophil ($r=0.84;p<0.01$) and mononuclear leucocyte infiltration ($r0.54=;p<0.05$) in cag A positive H.pylori infected patients. It was concluded that cag A –positive strains of H.pylori are associated with higher bacterial density, greater histopathological changes and increased systemic and local IL-18 production. This enhanced inflammatory response is thought to have role in disease pathogenesis, explaining the occurrence of peptic ulcer and sever gastritis with cag A positive strains.

Introduction

Helicobacter pylori (H.pylori) is a pathogenic microaerophilic spiral Gram- negative bacterium which plays a significant role in the pathogenesis of chronic gastritis, peptic ulcer and gastric cancer(Blaser and Parsonnet,1994). About 80% of H. pylori infections do not result in clinically relevant disease, even though such infections are often persistent and can be life long in many individuals (Blaser,1998). However, only a small number of infected individuals will suffer from clinically overt gastroduodenal disease and the

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reasons for this is unclear (Jenks et al.,1998). It has been proposed that certain strains of H. pylori may be more virulent and a variety of strain charact - eristics associated with disease severity have been identified ((Blaser, 1997). One Key marker is cytotxin- associated antigen (cag A), a high molecular weight (120-140 Kda) immunodo - minant protein encoded by the cag A gene.Cag A gene is an important marker for cag pathogenicity island(PAI) which is associated with an increased inflammatory response at gastric mucosal level (Peters et al., 2001). Althogh little is known about cag A function but its presence is found to be associated with increased mucosal cytokine production (Yamaoka et al.,2001). Cytokines are suspected to play a crucial role in the pathogenesis of H.pylori associated gastric disease(Hida et al.,2001).Interleukin-18 (IL- 18) is a recently identified proinflammatory cytokine produced mainly by macrophages .It stimulates interferon gamma production by natural killer cells and T cells (Kawabata et al., 2001). It was reported that Il-18 may play a role in gastric mucosal damage, but there is no much data available regarding its association with H.pylori infection and its cag A statuts (Crabtree et al.,1998).

Aim: The aim of this study is to investigate the H.pylori cag A status and its relationship to endoscopic and histopathological findings, as well as local and systemic IL-18 production in patients with dyspepsia.

Subjects and methods

Forty subjects with dyspeptic symptoms presented to Internal Medicine Department outpatient clinic at AL- Zahraa University Hospital were included in this study. They were 30 males and 10 females. Their ages ranged from 22 to 58 years old. All patients were subjected to full medical history, clinical examination, abdominal ultrasonography and electrocardiogra - phy. Non of the patients had previously received H.pylori eradication treatment, acid suppression treatment or non-steroidal anti-inflammatory drugs in the preceding 3 months. Patients with diabetes mellitus. hepatic, pancreatico - biliary , renal, and cardiac diseases were excluded. Five ml of blood were withdrawn from all patients, were left to clot and sera were separated for assay of routine laboratory tests (fasting blood glucose, urea , creatinine, serum transaminases (ALT and AST), bilirubin, alkaline phosphatase, albumin and serum amylase using chemistry autoanalyzer (Hitachi 911)) and serum IL-18 assay.

All patients were subjected to upper gastrointestinal endoscopy (Olympus ,GIF 240,Japan) for dyspeptic symptoms. Three antral biopsy specimens were obtained and used for:

1- Culture of H. pylori : one biopsy specipmen was placed in thioglycholate broth and transferred to the laboratory where it was homogenized by tissue homogeniser , cultured on Helicobacter selective medium (Colombia blood agar base and Dent supplement (SR147E),Oxoid .UK.) and incubated at 37º C under micro-aerophilic conditions using anaerobic jar and gas generating kit (Campylobacter gas generating kit, Oxoid , UK) for 3-7 days. Colonies that exhibited characteristics of colonial morphology of Helicobacter pylori were identified by Gram stained films and positive catalase, urease and oxidase reactions (Cheesbrough,2000).

2- Histopathological examination: Sections from formalin- fixed biopsy specimens were stained with haemato -

xlin and eosin and with Giemsa stain . The density of H.pylori bacteria , neutrophils (activity) and mononuclear (MNL) leucocytes were evaluated and graded from zero to 3 corresponding to non, mild, moderate and severe accor - ding to the updated Sydney system and using visual analogue scale (Dixon et al.,1996).

3- Assay of H.pylori specific gene and cag A gene by polymerase chain reaction (PCR) : one biopsy specimen was placed in phosphate buffered saline(PH 7.4) and was homogenized using ultrasonic homogeniser (Cole-parmer) and centrifuged at 8000 g for 10 minutes. The supernatent was separ - ated and protein content was determined for standerdization of IL-18 (Total protein in urine and CSF kit,Spinreact , Spain).The supernatants were stored at- 20º C till time of IL- 18 assay.

The DNA was extracted from the remaining pellet, which was resusp - ended in Tris- HCL buffer (PH 8) and proteinase k(0.5 mg/ml), incubated at 56º C for 1 hour, then boiled for 10 minutes(Broutet et al ., 2001). The extracted DNA was stored at- 20º C till assy of H.pylori specific gene and cagA gene by PCR.

Amplification of H.pylori rDNA :HS1/HS2 set of primers specific to the 16S rDNA of H.pylori was designed to amplify a 400bp fragment.

Amplification was carried out in a total volume of 50 ul containing 7ul of extracted DNA, 1x PCR buffer(10mM Tris/HCL, 50mMKCL,,1.5 mM MgCL), 2U Taq, 1ul of DTNPs (200 uM of each), 0.5uM of each primer (the HS1 primer AACGATGAAGCTTCTAGC TTGCTAG and the HS2 primer, GTGCTT ATT C- GTTAGATAC CGTCAT(Gulf Biotech,SA)) .The react -ions were performed using Biometra thermal cycler.The cycling conditions consisted of 40cycles as follows: 1minute at 94º C, one and half minute at 60º C and 1 minute at 72º C. The PCR products were separated by 1.5% agar - ose gel electrophoresis and stained with ethedium bromide.One hundred bp ladder (100-1600bp) marker (Pharmacia ,Sweden) was included in the gel. The PCR products were visua -lized by UV transilluminator (Avenaud et al,. 2000).

Amplification of cag A genomic DNA was carried out in a total volume of 50ul contaning 5 ul of extracted DNA, 1x PCR buffer (50mMKCL, 10mM Tris-HCL(PH8.3),1.5 mM $MgCL_2$) ,200 uM each nucleotide , 2 U of Taq and 0.5uM of each primer (sense, GATAACGCTGTCGCTT CATACG and antisense, CTGCAAA AGATTGTTTGGCAGA)(Gulf Biotech, SA).The reactions were performed using Biometra thermal cycler.The cycling conditions consited of 39 cycles as follows: 1min at 94º C, 1min at 55º C and 2min at 72º C.The PCR products were electrophorosed using 1.5% agarose gel and stained with ethedium bromide. A 100 bp ladder (100-1600 bp) marker(Pharmacia, Sweden) was included in the gel. The electrophoresis product was visualized with UV transilluminator.The cag A gene give 409 bp product(Danielsson et al.,2000).

4-Assay of IL-18 in serum and in supernatant of homogenized biopsy : was done using Human IL-18 ELISA kit, MBL, Japan. This kit measures IL-18 by sandwich ELISA, using 2 monoclonal antibodies against two different epitopes of human IL-18. The level of IL-18 was expressed as pg/ml in the serum and in tissue supernatant as pg/mg of biopsy protein .

Patients were determined to be H.pylori infected if positive by culture and or histological assessment and

positive for H.pylori specific rDNA by PCR.

Statistical analysis:

The data was analysed using the Statistical Package for Social Science (SPSS 8) and expressed as means ± SD. Paired-sample *t* test for comparison and Spearmann correlation and regression line analysis were used. P<0.05 was considered significant.

Results:

Forty dyspeptic patients were included in this study.Thirty of them (75%) were infected with H.pylori (positive culture , H.pylori in gastric mucosa and positive H.pylori specific rDNA (figure 1)). The cag A gene was detected in 16 out of the 30 H.pylori infected patients (53.33%)(figure 2). Ten out of 40 (25%) patients were negative for H.pylori by all methods of detection. Therefore according to our results, the forty dyspeptic patients were classified into 3 groups:

Group I: Sixteen out of 40 patients (40%) were H.pylori -positive and cag A- positive.

Group II: Fourteen out of 40 patients (35%) were H. pylori -positive and were cag A-negative.

Group III: Ten out of 40 patients (25%) were negative for H. pylovi by all methods of detection.

Macroscopic findings of endoscopy showed that in group I 7/16 (43.75%) of patients had duodenal ulcer and 9/16 (56.25%) had gastritis . In group II , all patients showed gastritis . In group III 6/10 (60%) of patients showed normal endoscopic findings and 4/10 (40%) showed gastritis (table 1).

Histopathological examinations of gastric biopsy specimens showed that the scores of H.pylori density and cellular infiltration (neutrophils and MNL) in gastric mucosa were significantly higher in cagA-positive patients (groupI) than in cagA -negative patients (groupII)(table 2). H.pylori density and MNL infiltration in gastric mucosa showed significant increase in group II as compared to their scores in group III , while neutrophil score showed non significant differe -nce(p>0.05) (table 2). Patients with gastritis 9/16 (43.75%) in cag A – positive group showed higher activity (according to Sydney system) as comp -ared to those in cag A -negative patient group (mean ±SD of neutrophil score, 1.55±0.52 vs. 0.85±0.36 ;p<0.01).

Serum IL-18 level showed significant rise in cag A positive patients(group I) (mean±SD , 308.43 ± 43.75) as compared to its level in cagA-negative patients (groupII) (Mean ±SD ,182.64±61.29 pg/ml; p<0.001) and as compared to group III (mean±SD, 62.1±5.83 pg/ml, p< 0.001) (table 3). Also, serum IL-18 showed significant rise in group II as compared to its level in group III (p< 0.01) .Moreover, IL-18 in gastric tissue supernatant showed significant rise in group I (mean± SD, 80.43 ± 23.81)as compared to group II (mean ±SD, 61.78 ± 11.73 pg/mg ; p< 0.01) and as compared to group III (mean±SD, 38.4±2.01pg/mg ; p< 0.01) Also, tissue IL-18 showed significant increase in group II as compared to its level in group III (p <0.001) (table3).

Serum IL-18 showed significant positive correlation with gastric IL-18 in group I (r=0.951; p<0.01) and in group II (r=0.998; p<0.01) (table 4&figure 3).Gastric IL-18 showed significant positive correlation with H.pylori density , neutrophils and MNL in gastric mucosa in group I (table 4 & figures 4-6) and only with H.pylori density and MNL in group II (table 4).

Table (1): Endascopic findings of endoscopy in all patient groups.

Endoscopic findings	GroupI (n=16)	GroupII(n=14)	GroupIII(n=10)
Duodenal ulcer	7 (43.75%)	-	-
Gastritis	9 (56.25%)	14 (100%)	4 (40%)
Normal mucosa	-	-	6 (60%)

Table (2):The scores (Mean ± SD) of H.pylori density, neutrophils and MNL in gastric mucosa in dyspeptic patient groups.

Bioindices	Group I (n=16)	Group II (n=14)	GroupIII (n=10)
H.pylori density:			
Mean ± SD	1.7±0.7	0.78±0.42	0.0
t	6.53	6	
p	<0.001	<0.001	
Neutrophils:			
Mean ± SD	1.87±0.80	0.85±0.36	0.80±0.42
t	4.777	.557	
p	<0.001	>0.05	
MNL:			
Mean ± SD	1.5±0.51	0.85±0.53	0.4±0.25
t	6.128	6.312	
p	<0.001	<0.001	

P<0.05= significant.

Table (3):Comparison of IL-18 levels in serum (pg/ml) and in gastric tissue supernatant (pg/mg) in all patient groups.

Bioindices	GroupI (n=16)	Group II (n=14)	Group III (n=10)
Serum IL-18			
Mean±SD	308.43±43.75	182.64±61.29	62.1±5.83
t	8.237	5.172	
p	<0.001	<0.01	
Tissue IL-18			
Mean±SD	80.43±23.81	61.78±11.73	38.4±2.01
t	3.340	5.542	
p	<0.05	<0.001	

P<0.05 = significant

Table (4): Correlation between serum IL-18 and gastric Tissue IL-18 (T.IL-18) and between T.IL-18 and H.pylori density ,neutrophils and MNL in goups I and II of patients.

Bioindices	Group I (n=16)		Group III (n=10)	
	r	P	r	P
S.IL-18/ T.IL-18	0.951	<0.01	0.998	<0.01
T.IL18 / H.desity	0.720	<0.01	0.671	<0.01
T.IL-18 /neutrophils	0.843	<0.01	0.758	<0.01
T. IL-18 /MNL	0.544	<0.05	0.607	<0.05

r < 0.05 = significant.

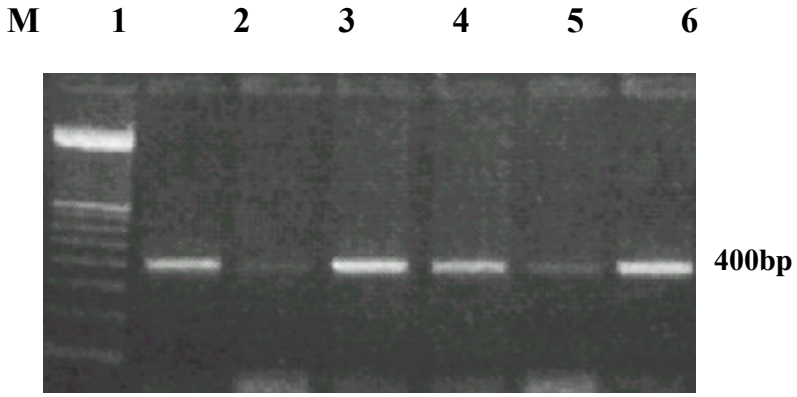


Figure (1): Ethidium bromide stained agarose gel demonstratig 400 bp PCR of H.pylori specific rDNA (lanes 1,3,4,6). M=molecular size marker

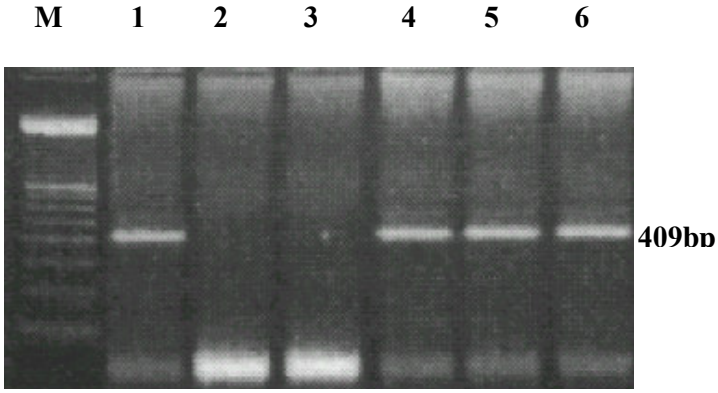
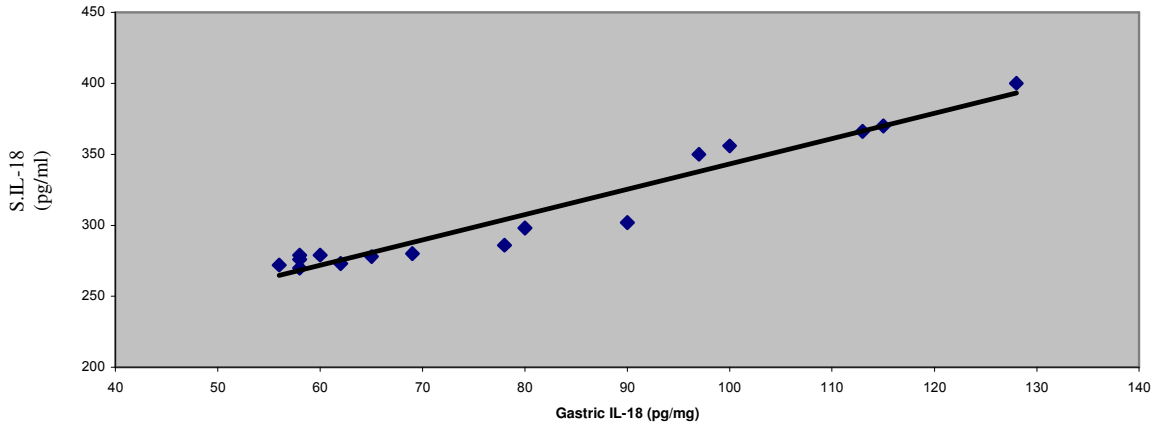
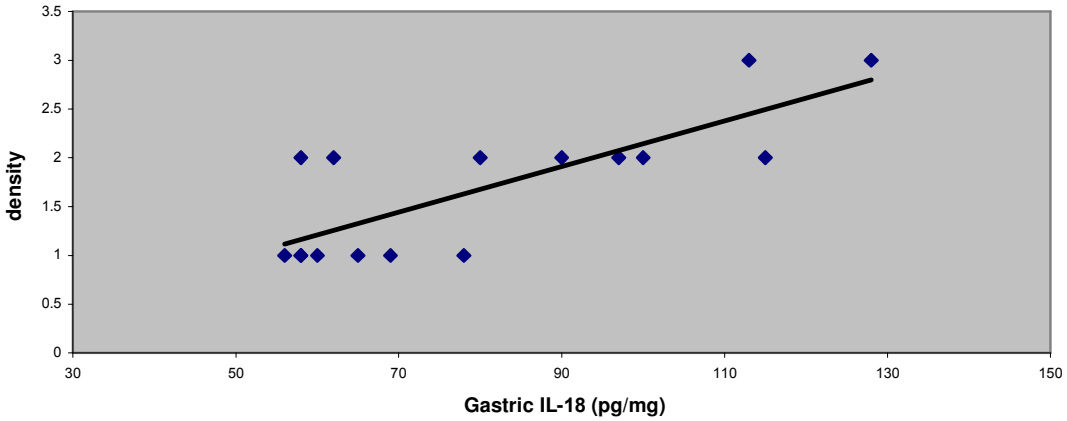


Figure (2): Ethidium bromide stained agarose gel with 409 bp band product of H.pylori cag A gene(lanes1,4,5,6). M= molecular size marker

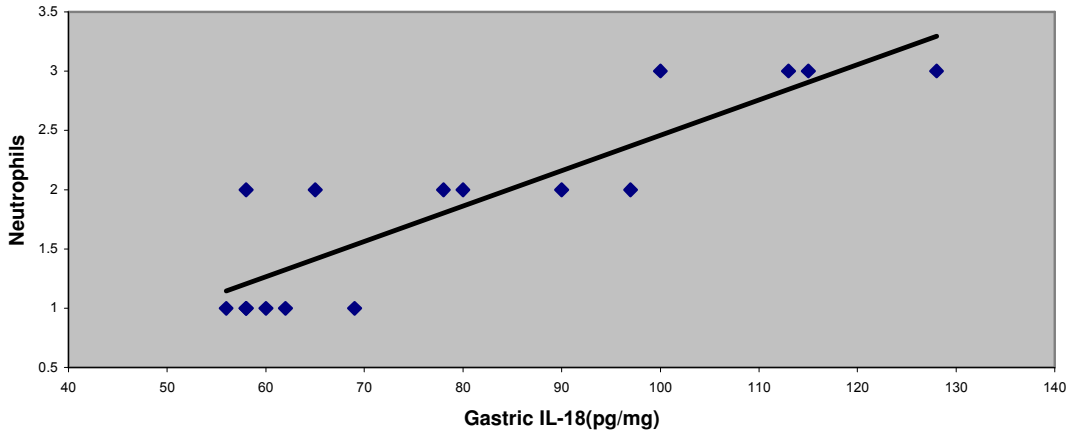
Figure(3): Correlation between s.IL-18 and gastric IL-18 in group I of patients



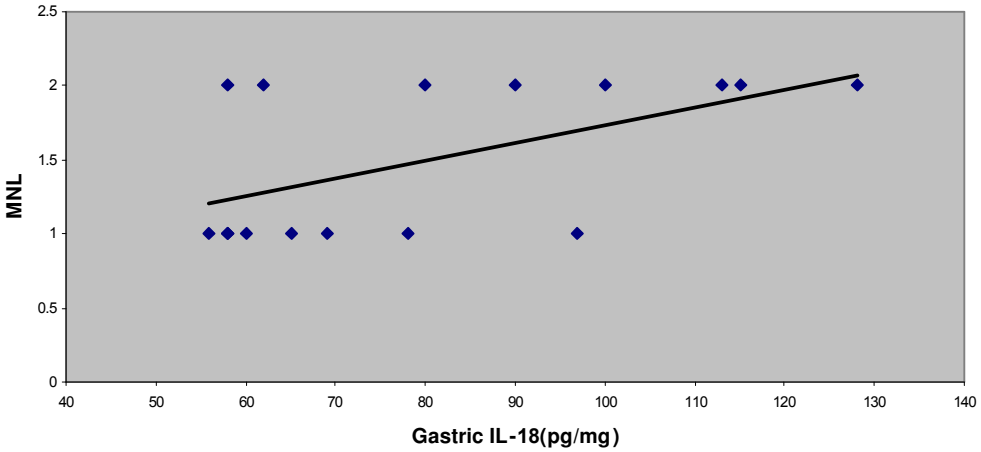
Figure(4): Correlation between gastric IL-18 and H.pyolri density in group I of patients



Figure(5):Correlation between gastric IL-18 and neutrophi score in group I of patients



Figure(6):Correlation between gastric IL -18 and MNL in group I of patients



Disscusion

Millions of people annually experience H.pylori associated disease that most often presents as chronic gastritis, peptic ulcer disease, gastric adenocarcinoma and mucosa associated lymphoma (McGowan et al.,1996 and Joyce et al., 2001). Many studies have suggested that some strains of H.pylori are more virulent than others and that cag A gene is a virulence factor for H.pylori (Queiroz et al., 2000 and Park et al., 2001). There is marked global variation in the frequency of cag A – positive strains in many populations(Web et al., 1999). Lag et al.,(1995) found that cag A gene is present in about 60-80% of H.pylori strains, while,Peters et al ., (2001) reported that about 50-60% of H.pylori strains were cag A positive. In the present study, among the 30 H.pylori –positive patie - nts, 16 (53.3%) were cag A- positive .

Many studies have been taken to investigate whether H.pylori cagA – positive strains differ in virulence and behaviour than cag A-negative strains (Tomita et al., 2001).The current study, revealed that in patients infected with cag A positive strain (group I), 7/16 (43.75%) had duodenal ulcer and 9/16 (56.25%) had gastritis. On the other hand, all patients of cag A –negative group had gastritis (table 1). These findings are parallel to those obtained by Hamlet et al., (1999) and Stone et al., (2001), who reported that cag A - positive strains were more frequent in peptic ulcer disease , gastric cancer and severe gastritis than in chronic gastritis. In the present study, patients infected with cag A positive strains showed significant increase in H.pylori density ($p<0.001$) and cellular infiltration (neutrophils ($p<0.01$)and MNL ($p<0.001$)) of gastric mucosa than in patients with cag A- negative strains (table 2). In agreement with our results, those obtained by Graham and Yamoaka (2000),who reported that the severity of gastric inflammation is rela - ted to the density of cag-A positive H.pylori in gastric mucosa.In addition, Saruc et al.,(2001) found greater histol - ogical changes in gastric biopsies obtain -ned from patients infected with cag A-positive H.pylori than those with cag A-negative strains. They also suggested that the virulenece of cag A-positive strains acts primarily as an accelerator in the disease and not predective of out come.

Moreover, gastritis in cag A-positive patients (group I) showed significantly higher activity as compared to that in cag A-negative patients (groupII) ($p<0.01$) . This was in accordance to Cox et al., (2001).

It has been found that immune response to H.pylori is considered to be a major factor contributing to gastric mucosal damage and that chronic H.pylori infection is accompanied by gastric mucosal production of IL-12 and IL-18 ,which promotes Th1 responses and interferon-γ production (Tomita et al.,2001,). In this study we found significant increase in serum($p<0.001$) and gastric tissue ($p<0.05$) levels of IL-18 in cag A-positve as compared to cag A negative patients and to non H.pylori infected patients ($p<0.001$)(table 3). These results are parallel to those obtained by Orsini et al., (2000) who found that cag A-positive H.pylori infection is associated with enhanced IL-18 expression than cag A-negative H.pylori infected patients with chronic gastritis and duodenal ulcer.

In the present study, serum IL-18 level showed significant positive correlation with gastric IL- 18(table 4). Moreover, gastric IL-18 showed significant positive correlation with H.pylori density, neutrophils and MNL scores in cag A- positive patients,

(table 4). Previous studies demon - strated significant positive correlation between mucosal pro-inflammatory cyt -okines as IL-6 and IL-8 with H.pylori density and cellular infiltration in cag A-positive strains of H.pylori (Ando,2000).

In conclusion, Cag A-positive strains of H.pylori are associated with greater bacterial density , inflammatory infiltrates in gastric mucosa and increa - sed systemic and local IL-18 production in patients with dyspepsia . This enhan - ced inflammatory response is thought to have a role in disease pathogenesis, explaining the occurrence of peptic ulcer and severe gastritis with cag A-positive than cag A –negative strains.

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"دراسة حالة الكاج أ للميكروب الحلزوني المعدي و الأنترلوكين –18 في مرضى عسر الهضم ."

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يعتبر الميكروب الحلزوني المعدي من البكتريا السالبة الجرام التي تسبب الأمراض المعدية المختلفة ، و هو ميكروب واسع الانتشار على مستوى العالم أجمع ، و يعتبر الكاج أ عامل من أهم العوامل اللازمة لضراوة الميكروب .ولقد عنيت هذه الدراسة ببحث العلاقة بين إيجابية وجود الكاج أ في الحالات الإيجابية للميكروب الحلزوني المعدي مع نتائج المنظار المعدي وتحليل الأنسجة . و كذلك قياس مستوى الأنترلوكين – 18 في المصل والأنسجة المعدية .و تشمل هذه الدراسة أربعين مريضا يعانون من عسر الهضم حيث تم أخذ عينات دم وعينات نسيج معدى بواسطة المنظار المعدي العلوي من كل مريض .وتم قياس مستوى الأنترلوكين – 18 في مصل هؤلاء المرضى و كذلك في النسيج المعدي .و تم أيضا الكشف عن وجود الميكروب الحلزوني المعدي في الأنسجة المعدية عن طريق فصل الميكروب و زراعته معمليا و الكشف عن حامضه النووي ، و كذلك عن وجود جين الكاج أ بإختبار تفاعل البلمرة المتسلسل .و قد ثبت وجود جين الكاج أ في 16 مريضا (40%) و قد ثبت أيضا وجود زيادة ذات دلالة إحصائية في مستوى الأنترلوكين – 18 في المصل و الأنسجة المعدية للمرضى الإيجابيين لوجود الميكروب الحلزوني المعدي و جين الكاج أ .و ظهرت هذه الزيادة الإحصائية واضحة فى المرضى الإيجابيين لوجود جين الكاج أ .كما ثبت أيضا وجود علاقة إيجابية هامة بين مستوى الأنترلوكين –18 في النسيج المعدي و كم البكتريا الحلزونية و الرشح الخلوي في المعدة ونستخلص من هذه الدراسة أن البكتريا الحلزونية الإيجابية لجين الكاج أ مصحوبة بكم أكبر من البكتريا و الاختلافات النسيجية المرضية و زيادة مستوى الأنترلوكين –18 الدوري و الموضعي . و يعتقد أن رد الفعل الالتهابي الزائد له دور في تكون المرض ، مما قد يفسر حدوث قرحة الأثنى عشر والتهابات المعدة الشديدة المصاحبين لنوعيه الكاج أ الإيجابية .