Association of the CagA Gene Positive Helicobacter pylori and Tissue Levels of Interleukin-17 and Interleukin-8 in Gastric Ulcer Patients

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It has been reported that CagA gene positive Helicobacter pylori (CagA+ H. pylori) induces severe gastric mucosal inflammation. On the other hand, Interleukin (IL)-17 is known to stimulate IL-8 release by the gastric epithelial cells which facilitates chemotaxis of neutrophils through an IL-8-dependent mechanism. The aim of the study is to determine the role of IL-17 and IL-8 in the development of gastritis and gastric ulcer in H. pylori infected patients. Mucosal biopsy samples were obtained from the ulcer site of gastric mucosa of 28 patients with gastric ulcer (GU), 27 with gastritis and 8 controls subjects without gastritis or ulcers. Infection with H. pylori of patients and controls was assessed by a rapid urease test, histological examination and culture. Measurement of the tissue levels of IL-17 and IL-8 were assayed by ELISA. H. pylori cagA gene was assessed by polymerase chain reaction (PCR). Out of the 28 patients with GU, 18 (64.2%) patients were positive for H. pylori infection, while 13 (48.1%) patients with gastritis and none of the controls were positive for H. pylori infection. The CagA gene was detected in 12 (66.6%) in H. pylori GU patients, and 7 (53.8%) H. pylori positive gastritis. IL-17 was significantly higher in GU-CagA+ve H. pylori compared to GU-CagA- H. pylori (P <0.05), while IL-8 showed no significant difference between groups. The mean levels of IL-8 in gastritis-CagA+ H. pylori was significantly higher compared to gastritis--CagA- H. pylori- (P <0.05). IL-17 showed significant association with the number of neutrophils in both GU and gastritis (r = 0.689, P < 0.05 & r = 0.618, P < 0.05). Also, IL-8 showed significant association with the number of neutrophils in both GU and gastritis n (r = 0.468, P < 0.05 & r = 0.727, P < 0.05). It is concluded that the Cag+ve H. pylori is associated with induction of mucosal injury. Also, IL-8 and IL-17 plays a role in the development of GU and gastritis especially in CagA+ H. pylori.

H. pylori, a microaerophilic, Gram-negative bacterium that selectively colonizes the human stomach, is the major cause of chronic active gastritis and peptic ulcer disease (Marshall & Warren, 1984; Graham et al., 1991; Blaser, 1999; Kabir, 2009).

IL-17, originally identified as cytotoxic T lymphocyte-associated antigen 8 (Rouvier et al., 1993), is a proinflammatory cytokine derived mainly from activated T cells (Chabaud et al., 1999; Aarvak et al., 1999; Chabaud et al., 2001). A number of studies discovered related molecules form a family of cytokines, the IL-17 family, and have been designated as IL-17A-F, with the prototype member being IL-17A (Rouvier et al., 1993; Spriggs, 1997; Shin et al., 1998; Moseley et al., 2003; Witowski et al., 2004; Huang et al., 2004; Hwang et al., 2004). IL-17A is produced exclusively by CD4+ activated T cells (Rouvier et al., 1993, Yao et al., 1995) and more specifically by CD4+CD45RO+ memory T cells (Shin et al., 1998; Kennedy et al., 1996). The human IL-17A gene product is a protein of 155 amino acids which is secreted as a disulfide-linked homodimer of a 30-35-ku glycoprotein (Fossiez et al., 1996; Fossiez et al., 1998). IL-17 exhibits pleiotropic biological activities on various cell types,
including macrophages, fibroblasts, endothelial and epithelial cells (Yao et al., 1995). IL-17 has been found to stimulate the synthesis of IL-1, IL-6, TNF, PGE2, ICAM-1 and cyclo-oxygenase-2 (Fossiez et al., 1998). Thus, IL-17 appears to provide a link between T cell activation and inflammatory responses. Consistently, an enhanced expression of IL-17 has been documented and implicated in the pathogenesis of immune-mediated diseases, such as rheumatoid arthritis, multiple sclerosis, and psoriasis (Fossiez et al., 1998). Moreover, IL-17 has the ability to stimulate IL-8 production in both epithelial cells and macrophages, raising the possibility that this cytokine may play an important role in the recruitment of inflammatory cells during bacterial infections (Laan et al., 1999).

In *H. pylori* infection, neutrophils are present within epithelial glands and the underlying lamina propria, and an increase in chronic inflammatory cells such as lymphocytes, macrophages, eosinophils, and plasma cells is also found in the lamina propria (Crabtree et al., 1991; Dixon, 1994; Ando et al., 1996; Ando et al., 1998). Studies have shown that *H. pylori* produces various cytokines that are related to neutrophil or mononuclear cell accumulation, such as IL-1β, IFN-γ, IL-6, IL-8, TNF-α and MIP-1α (Ando et al., 1996; Ando et al., 1998; Yamaoka et al., 1998; Kusugami et al., 1999). Among these cytokines, IL-8, the major human PMN chemoattractant, plays an important role in *H. pylori*-associated acute inflammatory responses (Ando et al., 1996, Ando et al., 1998, Shimizu et al., 2000). It was reported that IL-8 activity increases at ulcer sites (Shimizu et al., 2000), a finding which may be important to gastric ulcerogenesis. Ando et al., 1996, previously reported that IL-8 mRNA expression is often detected in gastric biopsy tissues from *H. pylori*-positive patients, but rarely in those from *H. pylori*-negative controls, indicating that gastric mucosa is an active site for the synthesis of both IL-17 and IL-8 in *H. pylori*-infected gastric ulcer patients (Mizuno et al., 2005).

Mizuno et al., 2005, showed that IL-17 and IL-8 activities in organ cultures of biopsy specimens from the antrum are substantially higher in *H. pylori*-positive patients than in *H. pylori*-negative patients, and that among *H. pylori*-infected gastric ulcer patients, the gastric mucosa at the ulcer sites contains significantly more IL-17 and IL-8 than the mucosa of the antrum.

A strong association was observed between infection with the CagA+ *H. pylori* strain and peptic ulcer disease, moreover, higher serum levels of inflammatory cytokine IL-18 in *H. pylori*-infected patients, especially in individuals infected with CagA+ strains (Jafarzadeh & Sajadi, 2006).

We aimed to study levels of IL-17 and IL-8 secreted at the ulcer site of *H. pylori* infected patients, and whether these levels differ between patients with CagA+ and Cag-ve *H. pylori*.

**Materials and Methods**

Mucosal biopsy samples were obtained from the gastric mucosa at ulcer site in 28 patients with gastric ulcer (GU), 27 with gastritis but no ulcers and 8 control subjects who were presented by dyspepsia and were found to have no gastritis and no ulcers. Patients were recruited from Mansoura University Hospital during upper gastroduodenal endoscopic examination from January 2010 to August 2010. None of the patients took any non-steroidal anti-inflammatory drugs, antibiotics or bismuth compounds during the preceding 3 months.

*H. pylori* infection was diagnosed when at least 2 of the following 3 tests were positive namely, bacterial culture, the rapid urease test, and identification of the organism in the Giemsa-stained sections in the gastric samples. Absence of *H. pylori* was defined by negative results of all the 3 tests.

Three antral specimens were taken endoscopically from adjacent areas of GU, gastritis and control patients, one for bacterial culture, rapid urease test (CLO test, Delta West, Bentley, Australia) and one for
histological examination, and the last for IL-17 and IL-8 measurement. Informed consent was obtained from all patients and the study protocol was approved by the ethical committee at Mansoura university hospital.

Gastric biopsies were homogenized using a tissue homogenizer. The protein was determined by commercial method (mg/100 mL). Homogenates were divided into portions of 200 μL each, then frozen and stored at -20°C for further examinations. The concentrations of IL-8 and IL-17 in homogenates of the gastric mucosa were determined by ELISA method. The results were expressed in milligram of protein in homogenate of the tissue examined (Maciorkowska et al., 2005).

Histopathological Assessment

Gastric biopsy specimens which were fixed in 10% formol saline embedded in paraffin and processed for histological evaluation. Sections (4-5μm) were cut and stained with Giemsa stain. The numbers of neutrophils and mononuclear cells infiltrating the lamina propria were counted in five high-power fields (Mizuno et al., 2005).

Culture and Identification

Homogenized tissue was cultured on Columbia blood agar. The plates were incubated at 35 ºC for 5-7 days under microaerophilic condition using (Campygen CNO 25A packets) inside a jar. Identification was based on morphological appearance using Gram stain, oxidase, catalase and urease tests.

Rapid Urease Test

This test was done for the biopsies and for colonies isolated from H. pylori selective agar media. The material was inoculated into Christensen's urea broth, urea broth base with 40% sterile urea solution (Oxoid, UK). Change in color from yellow to purple-red was observed after 30 minutes and after 2 hours.

Measurement of IL-17 by ELISA

The RayBio® Human IL-17 ELISA kit was used. One hundred μl of standards, samples were added to the appropriate microtiter wells. After incubation for 2.5 hours, wells were aspirated and washed. One hundred μl biotinylated antibodies were added to each well and incubated for 1 hour and thereafter, wells were thoroughly aspirated and washed. One hundred μl of TMB one step substrate reagent were added to each well. After incubation for 30 minutes, 50 μl of stop solution were added to each well. Absorbance of each well was read at 450 nm (using ELISA reader Spectra III, Austria) having blanked the plate reader against a chromagen blank.

Measurement of IL-8 by ELISA

Human IL-8 immunoassay: (Quantikine, R&D system, Minneapolis, U.S.A.) was used. One hundred μl assay diluent were added to each well. Fifty μl standard, control or samples were added to each well, incubated for 2 hours at room temperature. After wash, 100 μl conjugate were added to each well, incubated for 1 hour at room temperature. After wash, 200 μl substrate solution were added to each well, incubated for 30 minute at room then, 50 μl stop solution were added to each well, read at 450 nm ºC

DNA Isolation

QiAamp DNA mini kit (QIAGEN Inc., Valencia, CA, USA) was used. Biopsy specimens was diluted with 1 ml of 0.9% sodium chloride and mixed well by vortexing and was put into microfuge tube and centrifuged at 7500 rpm for 10 min. and the supernatant was discarded and 180 μl buffer ATL were added to the pellet. Twenty μl Proteinase K were added and mixed by vortex. After incubation at 56 °C for 1 hour, 200 μl of buffer AL were added and mixed by pulse-vortexing for 15 seconds and incubated at 70 °C for 10 min. 200 μl ethanol 100% were added to the sample and mixed by pulse-vortexing for 15 seconds and the mixture was applied to QIAamp spin column and centrifuged at 8000 rpm for 1 min. 500 μl buffer AW1 were added to the QIAamp spin column and centrifuged as before. 500 μl buffer AW2 were added to the QIAamp spin column and centrifuged at 14000 rpm for 3 min. 200 μl buffer AE were added to the QIAamp spin column and incubated at room temperature for 5 min. and then centrifuged at 8000 rpm for 1 min. and the elute was collected in sterile tubes and stored at -20 °C till PCR reaction was done.

PCR

A 349-bp target sequence from CagA gene was amplified using two primers: P1(5’-GATAACAGGCA AGCTTTTGAGG-3’) and P2(5’- CTGCAAAAGATT GTTGCAGGA-3’) (Peek, et al., 1995).

The PCR was performed in a total volume of 50 μl of master mix (EzWay PCR master mix, Koma Biotech, Korea) containing 1μl of the extracted DNA and 0.5 μM of each primer. The samples were amplified in a thermal cycler (Norwall, CT, USA). Cycling conditions were 94°C, 55°C, and 72°C for 1 min at each step for 40 cycles. A final extension was done for 7 min at 72°C then temperature was held at
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Statistical Analysis

It was carried out via both Statistical Package for Social Science (SPSS) version 17. Qualitative data were represented in the form of number and frequency, while quantitative data were represented in the form of mean ± standard deviation (mean±SD). Kolmogrov-smirnov test was used to test normality of quantitative data, and all data were normally distributed. Student’s t test was used to compare groups, while Pearson’s correlation test was used to determine correlation between variables. All tests were considered significant if $P \leq 0.05$.

Results

Out of the 28 patients with GU, 18 patients (64.2%) were positive for H. pylori infection, while 13 patients (48.1%) were positive for H. pylori infection out of 27 patients with gastritis and we could not confirm H. pylori infection in 8 control persons.

Cytokine Assay

The mean levels of IL-17 and IL-8 in GU (20.75±5.778 pg/ml & 64.79±14.06 pg/ml) and gastritis (8.852±4.185 & 19.41±10.26 pg/ml) were significantly higher when compared to control groups (0.0 & 1.125±2.1 pg/ml) ($P^{**}<0.05$ and $P^{***}<0.05$ respectively) (Table 1).

The CagA gene was detected in 12/18 (66.6%) H. pylori GU patients, and 7/13 (53.8%) H. pylori gastritis. Regarding cytokine levels, the mean levels of IL-17 and IL-8 in GU (CagA+ H. pylori) (26.25±2.864 pg/ml & 78.42±7.856 pg/ml), IL-17 were significantly higher compared to GU (CagA- H. pylori) (20.00±0.894 & 59.17±6.274 pg/ml), while IL-8 showed no significance.

The mean levels of IL-17 and IL-8 in gastritis (CagA+ H. pylori) (15.4±1.345 & 32.71±9.945 pg/ml) showed significant difference in IL-8 level compared to gastritis (CagA- H. pylori) (8.833±0.753 & 18.67±1.633 pg/ml) ($P^{###}<0.05$) while IL-17 showed no significant difference ($P^{###}=0.223$) (Table 2).

Cytokine Levels and Histological Findings

Comparison of mean numbers of neutrophils and mononuclear cells infiltrating lamina propria in GU (142.3±20.32 & 519.1±43.81) and gastritis (47.19±26.82 & 292.6±48.87) showed significant difference in mononuclear cells and when compared with control groups (1.375±2.065 & 97.87±67.70). Significant difference was also detected between GU and gastritis versus controls regarding neutrophils (Table 3).

<table>
<thead>
<tr>
<th>Table 1. IL-17, IL-8 levels in H. pylori infected patients with gastric ulcers, gastritis and controls.</th>
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<tbody>
<tr>
<td><strong>IL-17 (pg/ml)</strong></td>
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<tr>
<td>Mean±SD</td>
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<tr>
<td>GU</td>
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<td>Gastritis</td>
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<td>Control</td>
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<td>$P^*$</td>
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<td>$P^{**}$</td>
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$P$ (GU vs. gastritis), $P^{**}$ (GU vs. control), $P^{***}$ (gastritis vs. control).

GU= gastric ulcer. $P<0.05$ IS significant. NS= not significant.
Table 2. Comparison of IL-17 and IL-8 levels between patients with CagA+ and CagA- *H. pylori* infection.

<table>
<thead>
<tr>
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<th>IL-17 (pg/ml)</th>
<th>IL-8 (pg/ml)</th>
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<tr>
<td></td>
<td>Mean±SD</td>
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<td>GU (CagA+ <em>H. pylori</em>)</td>
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\( P^* \) < 0.05
\( P^{**} \) NS
\( P^{***} \) NS
\( P^# \) < 0.05
\( P^{##} \) NS <0.05
\( P^{###} \) NS < 0.05

\( P^* \) GU (CagA+ *H. pylori*) vs GU (CagA- *H. pylori*), \( P^{#} \) (GU (CagA+ *H. pylori*) vs. Gastritis (CagA- *H. pylori*), \( P^{##} \) GU (CagA+ *H. pylori*) vs. Gastritis (CagA- *H. pylori*), \( P^{###} \) GU (CagA- *H. pylori*) vs. Gastritis (CagA+ *H. pylori*).

GU= gastric ulcer. \( P<0.05 \) is significant. NS= not significant.

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Table 3. Neutrophils and mononuclear cells infiltrating lamina propria in gastric ulcer, gastritis and control groups.

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<thead>
<tr>
<th></th>
<th>Neutrophils</th>
<th>Mononuclear cells</th>
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<tr>
<td></td>
<td>Mean±SD</td>
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\( P^* \) (GU vs. gastritis), \( P^{**} \) (GU vs. control), \( P^{***} \) (gastritis vs. control).

GU= gastric ulcer. \( P<0.05 \) is significant. NS= not significant.
The association between cytokine levels and histological findings was investigated by quantifying the number of infiltrating neutrophils and mononuclear cells. IL-17 showed a significant association with the number of neutrophils in both GU and gastritis ($r = 0.689$, $P < 0.05$ & $r = 0.618$, $P < 0.05$). Similarly, IL-8 showed significant association ($r = 0.468$, $P < 0.05$ & $r = 0.727$, $P < 0.05$). Regarding number of mononuclear cells, IL-17 ($r = 0.707$, $P < 0.05$ & $r = 0.761$, $P < 0.05$) and IL-8 ($r = 0.654$, $P < 0.05$ & $r = 0.711$, $P < 0.05$) showed a similar significant association. (Fig. 1, 2, 3).

**Figure 1.** Correlation between cytokine levels and neutrophils: a) neutrophils & IL-17 in gastric ulcer, b) neutrophils & IL-17 in gastritis, c) neutrophils & IL-8 in gastric ulcer, d) neutrophils & IL-8 in gastritis.
Figure 2: Correlation between cytokine levels and mononuclear cells: a) mononuclear cells & IL-17 in gastric ulcer, b) mononuclear cells & IL-17 in gastritis, c) mononuclear cells & IL-8 in gastric ulcer, d) mononuclear cells & IL-8 in gastritis.

Figure 3. H & E staining of gastric biopsy specimens a) normal gastric mucosa, neutrophils are absent, b) gastritis, numerous neutrophils and mononuclear cells appear in the lamina propria and c) gastric ulcer, neutrophils and mononuclear cells were infiltrating the lamina propria.
Discussion

Considerable evidence has been accumulated to indicate that IL-8 plays a major role in the Helicobacter-associated acute inflammatory response (Crabtree et al., 1994). Both IL-8 mRNA and protein levels strictly correlate with Helicobacter density (Shimoyama et al., 1998). In addition, IL-8 expression has been associated with significantly more severe infiltration of PMN, and down-regulation of mucosal IL-8 synthesis, induced by Helicobacter eradication, is paralleled by a resolution of the neutrophils infiltration (Ando et al., 1998). During Helicobacter infection, epithelial cells are the major producers of IL-8, even if there is evidence that macrophage-like cells synthesize IL-8 (Crabtree et al., 1994). Although several studies have documented the ability of Helicobacter to directly stimulate IL-8 synthesis, there is evidence that T cell-derived cytokines may modulate epithelial cell IL-8 gene expression (Yasumoto et al., 1992).

IL-17 is capable of modulating the expression of various genes in fibroblasts, macrophages, epithelial and endothelial cells and is implicated in the pathogenesis of human inflammatory diseases (Yao et al., 1995). Shin et al., (1999) reported that lamina propria mononuclear cells (LPMC) isolated from the human gastric mucosa express IL-17 at both RNA and protein levels.

In our study, the mean levels of IL-17 and IL-8 in GU and gastritis were significantly higher compared to control groups. Also, the mean levels of IL-17 and IL-8 in gastritis were significantly higher compared to control group.

It has been found that IL-17 stimulates IL-8 release by gastric epithelial cells and facilitates the chemotaxis of neutrophils through an IL-8-dependent mechanism, and contributes to the enhancement of IL levels in H. pylori-colonized gastric mucosa (Luzza et al., 2000) and has the ability to stimulate IL-8 production in fibroblasts (Hwang et al., 2004). Neutralization of IL-15 resulted in a significant inhibition of IL-8 production in gastric LPMC. These data thus support previous studies indicating the ability of IL-17 to induce IL-8 expression in other systems (Laan et al., 1999).

Vaccination of mice against H. pylori results in a significant Th-17 cell recall response associated with increases in chemokines that attract neutrophils to the stomach, which are important for eradication of H. pylori (DeLyria et al., 2009).

Although the mechanisms through which IL-17 enhances IL-8 have not been extensively investigated, it is known that IL-17 can activate transcription factors, such as NF-B and mitogen-activated protein kinases (Shalom-Barak et al., 1998) that are capable of initiating IL-8 expression (Yao et al., 1995; Fossiez et al., 1996). Mizuno et al., (2005) indicated that among H. pylori-infected gastric ulcer patients, a significant correlation was seen between IL-17 and IL-8 levels at each biopsy site.

When RNA transcripts for IL-17 were analyzed by a semi-quantitative RT-PCR, an increased accumulation was found in both mucosal tissue and LPMC samples from Helicobacter-infected patients compared with that in uninfected subjects. Similarly, the amount of IL-17 protein, as determined by Western blotting, was increased in the mucosa of Helicobacter-infected patients. In addition, Helicobacter eradication resulted in a down-regulation of IL-17 production. As no difference in terms of IL-17 expression was seen in blood lymphocytes isolated from patients with Helicobacter-infected patients and controls, it is highly likely that the mucosal microenvironment, determined by the bacteria infection, influences IL-17 expression (Luzza et al., 2000).
Our study showed that the mean numbers of neutrophils and mononuclear cells infiltrating lamina propria in GU were significantly higher compared to gastritis and control groups. Also, the mean numbers of neutrophils and mononuclear cells in gastritis were significantly higher compared to control group. IL-17 and IL-8 showed a significant correlation with the number of neutrophils and mononuclear cells.

At the ulcer site, however, IL-17 had significant correlation with mononuclear cells and neutrophils than IL-8, and a significant correlation with neutrophils than with mononuclear cells. IL-17 and IL-8 have been reported to cause neutrophil recruitment (Laan et al., 1999), and neutrophils are known to cause damage to tissues they infiltrate (Luzza et al., 2000). On the basis of these results, Mizuno et al. (2005) hypothesize that, together with IL-8, IL-17 might contribute to ulcerogenesis in gastric ulcer patients.

In our study, out of the 28 patients with GU, 18 patients (64.2%) were positive for *H. pylori* infection, while 13 Patients (48.1%) were positive for *H. pylori* infection out of 27 patients with gastritis and we could not confirm *H. pylori* infection in 8 control persons. The CagA gene was detected in 12 patients (66.6%) out of 18 *H. pylori* GU patients, and 7 patients (53.8%) out of 13 *H. pylori* positive for gastritis. Our work concur that of peek et al., (1995) who test the hypothesis that cagA+ strains elicit a greater proinflammatory cytokine response in the gastric mucosa than cagA- strains. They obtained gastric biopsies from 52 patients and were studied by histology, culture, ELISA, and RT PCR. Of 52 patients, 32 (62%) were infected with *H. pylori* based upon both serology and histology or culture, 16 (31%) were negative by serology, histology, and culture, and four (7%) were positive by serology only. Of 15 *H. pylori*-infected patients with peptic ulceration, 14 (92%) were infected with cagA+ strains compared with 8 (50%) of 16 patients with gastritis alone, and those infected with cagA+ strains had significantly higher grades of inflammation in the gastric mucosa indicating that infection with cagA+ *H. pylori* strains is associated with higher grades of gastric inflammation, correlating with enhanced mucosal levels of IL-8, and increased risk of peptic ulceration (peek et al., 1995).

Our results concerning cagA gene also support other studies but with some differences due to different study conditions. In another study, *H. pylori* strains from 35 patients [21 gastritis, 14 peptic ulcer diseases] were analyzed using PCR. The CagA gene was detectable in 57.1% of gastritis and 92.9% of peptic ulcer diseases-associated strains (Salih et al., 2007). CagA status was determined using an anti-CagA ELISA. Anti-CagA antibodies were more prevalent among dyspeptic patients with gastric cancer or peptic ulcer (73.3%) compared to those with non-ulcer dyspepsia (40%). The prevalence of CagA in Egypt was related to the clinical presentation of *H. pylori* infection being lowest in asymptomatic controls (11.1%) and increasingly prevalent in non-ulcer dyspepsia (40%), peptic ulcer (66.7%), and gastric cancer (89%) (Essa et al., 2008).

In addition, IL-17 was found significantly higher in GU (CagA+ *H. Pylori*) compared to GU (CagA- *H. pylori*). Also, the mean levels of IL-8 in gastritis (CagA+ *H. pylori*) were significantly higher compared to gastritis (CagA- *H. pylori*), and this is consistent with several previous studies. It has been shown that the mucosal levels of IL-6 and IL-8 were significantly higher in CagA+ *H. pylori*-infected groups in comparison to CagA- *H. pylori*-infected groups (Yamaoka et al., 1997; Ando et al., 2000). The main function of IL-17 is the induction of IL-8 from cells such as epithelial cells (Caruso et al., 2007). Accordingly, the differential effects of CagA+
strains and CagA– strains on IL-8 production may be attributable in part to their differential effects on IL-17 production. This may account for the induction of more severe gastric inflammation by CagA+ strains than by CagA– strains. Thus in DU patients, the IL-17 response was dependent on the CagA status of the infecting strains. These findings indicate that IL-17 serum levels are positively influenced by \textit{H. pylori}, particularly by CagA+ strains. However, since a proportion of DU patients are CagA–, various genetic host factors may also be important for the development of DU (Jafarzadeh \textit{et al.}, 2009).

References


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