IMMUNOBLOT ASSAY FOR THE DETECTION OF HELICOBACTER PYLORI ANTI-CagA & ANTI-VacA ANTIBODIES IN DYSPEPTIC PATIENTS

by

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MSc. Thesis

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KEYWORDS: *Helicobacter pylori,* CagA, VacA, Peptic Ulcer Diseases, ELISA, Immunoblotting.

ABSTRACT

H. pylori has been incriminated as a causative agent of peptic ulcer diseases. The possession of CagA & VacA virulence factors was found to play a role in the pathogenesis & the outcome of the disease.

The aim of the study was to detect anti-H.pylori antibodies in dyspeptic patients by the ELISA test, to detect the presence of anti-CagA & anti-VacA antibodies & to determine their association with peptic ulcer diseases (PUD).

Material & Methods: 45 patients (26M,19F) age 16-80 years (average 40) attended the "Okmeydanı & Samatya SSK" hospitals were undergone endoscopy. 5 antral biopsies were obtained for CLO, culture & histopathology. Serum sample from each patient was also collected.

Results: The ELISA test was (+) in 32 out of 39 patients that were CLO (+) & histopathology (+). The test showed 82% sensitivity, 83% specificity, 97% positive predictive value, & 42% negative predictive value.Western blot analysis detected a 116kDa (CagA), & 89kDa (VacA) bands. Only 3 patients (10%) were VacA (+).

Conclusion: The ELISA test was found to be useful for the detection of H.pylori antibodies. There wasn't a significiant association between the presence of anti-CagA antibodies & duodenal ulcer (85%), gastritis (79%), gastric cancer (100%) & gastric ulcer (100%), which further substantiate the role of CagA in the pathogenesis of PUD.

PEPTİK ULSER HASTALARINDA ANTİ-CagA ve ANTİ-VacA ANTİKORLARININ TARANMASINDA IMMUNOBLOT TEKNİĞİ

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ANAHTAR KELİMELER: *Helicobacter pylori,* CagA, VacA, Peptic Ulcer Diseases, ELISA, Immunoblotting.

ÖZET

Helicobacter pylori bakterisi peptik ülser hastalıklara yol açacıcı ajan olarak anılmaktadır. Virulens faktörler olarak CagA ve VacA antijenlerinin varlığının bu hastalıkların çıkışında ve patojenliğinde önemli bir rol aldığı bulunmuştur.

Çalışmanın amacı; peptik ülser hastalarında ELISA testi vasıtası ile anti-Helicobacter pylori antikorlarını ve immunoblot testi ile de anti-CagA ve anti-VacA antikorlarını taramak ve anti-CagA ve de anti-VacA antikorları ile bu hastalıklar arasındaki ilişkinin ne olduğunu belirlemekti.

Materyal ve Metod: Okmeydanı ve Samatya SSK hastanelerine gelen yaşları 16-80 arası olan (ortalama 40) 26'sı erkek, 19'u kadın 45 hasta endoscopiye tabi tutuldu. CLO, kültür ve de histopatoloji için olmak üzere her bir hastadan 5 tane antral biyopsi alındı. Ayrıca her hastadan serolojik tahliller içinde serum alındı.

Sonuçlar: ELISA testi, CLO ve histopatoloji testlerinin pozitif olarak gösterdiği 39 hastanın 32 sinde pozitifti. Test % 82 sensitiv, % 83 spesifik iken , testin tahmini pozitif değeri ile tahmini negatif değeri sırasıyla % 97 ve % 42 olarak bulunmuştur . Western blot testi ile 116 (CagA) kDa, ve 89 (VacA) kDa bandları tarandı. Sadece 3 hasta 89 kDa (VacA) positif çıktı.

Tartışma: ELISA testi H. pylori antikorları taramasında faydalı olduğu görüldü. Anti-CagA antikoru ile duodenal ülser (83 %), gastrit (%78), gastrik kanser (100%), ve gastrik ülser (100%) arasında kayda değer bir ilişti var oldadığı müşayede edildi , ki bir sonraki çalışmada CagA antijeninin Peptik ülser hastalıklarında ki nasıl bir patojenik etkiye sahip olduğunun araştırılması tavsiye edilmektedir.

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I. GENERAL INTRODUCTION

For most of the past century the cause of peptic ulcer disease was thought to be stress-related and the disease to be prevalent in hyperacid producer. The isolation of Helicobacter pylori from the stomach of patients with gastritis, and peptic ulceration and the suggestion for its association with peptic ulcer diseases was initially met with skepticism (4, 38). But later on research done in this area proved such association (38)

Infection with H. pylori was found to be ubiquitous. Over 50% of the world population is infected with H. pylori with high prevalence being in developing countries than in developed countries and increases proportionally with age (34,56). The organism was reported to be associated with chronic active type B gastritis, gastric ulcer, duodenal ulcer, gastric carcinoma and MALT lymphoma (2,22,46).

Helicobacter pylori is a gram negative, microaerophilic, spiral-shaped and flagellated bacteria that colonize the stomach of human being (4,36). The organisms possess several virulence factors; these are urea, flagella, heat shock protein, vacuolating cytotoxin, and CagA antigen (4).

Helicobacter pylori appeared to display genetic diversity and that certain strains are more pathogenic than others. H. pylori was divided into two types according to the presence or absence of cagA and vacA genes. Type I strains contain both cagA and vacA whereas Type II strains contain neither ones. These genes encode for CagA and VacA proteins that have been reported to be associated with peptic ulcer diseases (2,5,6). However reports from developed countries indicated that cagA gene expression was found not to be associated with an increased risk of developing peptic ulcer diseases (6,7,8).

Several tests have been applied for the diagnosis of H. pylori infection, these are divided into biopsy-based tests, (CLO, culture, histology, and PCR), and non biopsy-based tests, (serology, urease breath test) (20). Serology, which is cheap, non-invasive, rapid and easy to perform, has been widely used in

epidemiological and post-treatment studies (55). ELISA and latex agglutination are used for the detection of antibodies against H. pylori whereas immunoblot assay is used to detect antibodies against a specified antigen of the organism such as CagA and VacA antigens (53,55).

The aim of the study was to detect anti-H.pylori antibodies in dyspeptic patients by the ELISA test, to detect the presence of anti-CagA & anti-VacA antibodies by western blot & to determine their association with peptic ulcer diseases.

II. REVIEW OF LITERATURE

A) History

In 1979 Warren, a pathologist working at the Royal Perth Hospital in Perth, Western Australia, described a curved bacteria found in gastric biopsy specimens submitted for histopathologic examinations (4,10,36). He noticed that these bacteria were present in the mucus layer covering the gastric mucosa tissue (4,36). According to Marshall (36) Bizzozero, in 1893, and later on Salomon, in 1896, have wrote letters describing a spiral bacteria to be present in the stomachs of cats and dogs but less commonly in kittens (36). Freedberg and Barron 1940 noticed the presence of similar bacteria in gastric tissue from patients with peptic ulcer disease or cancer. Ten years later Palmer, a pathologist, denied the existence of such bacteria (36). Palmer's report supported the hypothesis that the stomach is a sterile organ and that bacteria cannot survive in gastric acid environment, and thus the existence of bacteria was subsequently overlooked by scientist (10, 36).

It was until 1974, when Steer and Colin-Jones redescribed the presence of bacteria on the surface of gastric mucosa in patients with gastric ulcer. The organisms were incorrectly identified as pseudomonas spp. (35).

In 1981 R. Warren at the Royal Perth Hospital in Western Australia first showed B. J. Marshall, a young trainee in internal medicine, the spiral bacteria he had discovered in 1979. Marshall became interested in Warren's observations and they made a decision to work on the organism and to isolate it from biopsy specimens. (4,10,35,36). They hypothesized that the spiral bacteria might belong to the Camplyobacter organisms (10) and named it *Camplyobacter pyloridis* (4), which was changed later to Camplyobacter pylori because of their location in the stomach (36). Further investigation regarding the phylogenetic properties of this showed that these organisms have a flat cellular surface, a highly strong urease activity, 4–6 polar sheathed flagella, a unique fatty acid profile, different respiratory quinines from complyobacters and a very different 16SRNA sequence which made

scientists be able to separate them from Camplyobacter and rename it as Helicobacter pylori in 1988 (4,15).

Scientists all over the world started to confirm the existence of H. pylori in the gastric mucosa (4). By the year 1984, it had already become clear that H. pylori infection was highly associated with the presence of inflammatory conditions, chronic superficial gastritis and chronic active gastritis (10). Marshal and Raven hypothesized that if there is a link between gastritis and H. pylori then, there might be an association with other gastritis-associated diseases, such as peptic ulcer and gastric cancer. (10) Later on, Marshall and Warren illustrated that there was a correlation between Helicobacter pylori infection and duodenal ulceration (36). This observation was confirmed and widened to include gastric ulceration (4,10,11). The International Agency for Cancer Research (IACR), an arm of the World Health Organization (WHO) reviewed the available facts and declared that Helicobacter pylori was a carcinogen of humans in 1994 (11,13). Also the current recommendation from the National Institutes of Health (NIH) Consensus Conference held in 1994 that all patients with gastric ulcer and Helicobacter pylori infection should be treated for Helicobacter pylori (11,13).

Helicobacter pylori causes chronic persistent gastritis in nearly all infected subjects, leading to the development of atrophic gastritis, intestinal metaplasia and eventually dysplasia and gastric cancer in a number of infected subjects. (2) H. pylori infection has been also linked to the gastric mucosa--associated lymphoid tissue lymphoma (MALToma) in which patients treated with antibiotics showed regression of the tumor and to the development of gastric non-Hodgkin's lymphomas (4).

B) Helicobacter pylori

1. Microbiology

Helicobacter pylori is a gram negative, microaerophilic, spiral, rod-shaped curved and flagellated bacteria (15,35). The organism is 2.5-5.0 μm in length and 0.5–1.0 μm in width. They may appear as a coccoid when cultured on solid or liquid media (4).

The organism is best grown in a moist microaerophilic atmosphere that contains 5–10% O_2 and 10% CO_2, and incubated at 37°C (4,13). Marshall et al. (1984) used moist chocolate agar and blood agar to isolate the organisms (35) Other media that are used to support the growth of the organism are Skirrow's medium, modified chocolate agar, triptycase soy agar, brucella agar, brain heart infusion agar, egg yolk emulsion agar, Dent's medium to colonize the bacteria (13).

Though bacteria are very sensitive to acidic environments, H. pylori has the ability to colonize the unique ecological niche at the surface of gastric epithelial cells within or beneath the mucous bicarbonate layer (16). It is indicated that bacteria can replicate at a pH of 4.3 whereas they can survive to a pH of 2.3 and do not grow over a pH of 7 (16). It is assumed that urease enzymes, which are produced by all known H. pylori isolates with large quantities, are very active to result in a neutral microenvironment (4,16). Further, H. pylori rarely invade the lamina propria and gastric cells because the bacteria in the stromal cell of laminar propria was observed. The invasion of H. pylori into the human gastric mucosa will more intensely stimulate the inflammatory cells to produce oxygen radicals that ultimately transform the chronic gastritis into gastroduodenal ulcers and gastric carcinoma (25).

H. pylori was shown to possess a putative outer membrane proteins (OMPs) of 31-80 kDa which enables the organism to adsorb urease and heat shock protein B (HspB), which are found strictly within the cytoplasm in early log phase cultures of Helicobacter pylori and released by autolysis, to its surface.

Furthermore four porin molecules, each of which forms pores, have been identified. (4)

The flagella has been found to play an important role in the colonization process. The FlaA and FlaB genes have been believed to act as regulators of the flagellar expression. Since it has been observed that bacteria carrying mutant FlaA and FlaB genes cannot colonize the gastric mucosa (16).

The biological activity of H. pylori LPS (lipopolysaccharide) is low. It means that it is less harmful for the host (4). Studies done on animals indicated that the lethal toxicity of H. pylori LPS in mice was 500-fold lower than that of salmonella LPS (17). Furthermore, several studies showed that the inflammatory response against H. pylori LPS were significantly lower when compared with that against Escherichia coli and salmonella LPS (17). The structure of the O-specific chain of H. pylori LPS in different H. pylori resembles that of host Lewis^x or Lewis^y blood group antigens expressed normally in human gastric mucosa. This mimicry enables the bacteria to camouflage and thus to escape the host immune defences (4, 17).

H. pylori possess nearly 1600 genes that have an average of 1.7 million nucleotides (18). Fifty five percent of the genes were identified in other organisms, the rest are unique to the strain (18). Some bacteria can transmit a piece of their DNA fragment to another bacterial strains or species (18). If the segment carries genes that codes for any virulence factor, this can be described as a pathogenicity island (PAI). H. pylori cagPAI was found on the circular shaped chromosome (18). Although approximately 40% of Helicobacter pylori isolates carry plasmids, it is believed that none of these plasmids carries any identified virulence factors (4).

2. Epidemiology

In regards to the prevalence of H. pylori infection, it was found that 50 - 70% of the population in developing countries carry H. pylori while only 25-50% of those people in developed countries were infected (4). An age difference was also noticed, approximately 20% of individuals below 45 years of age were infected

with H. pylori whereas 50% of those above the age of 60 years are carrying the bacteria (Figure 1) (15).

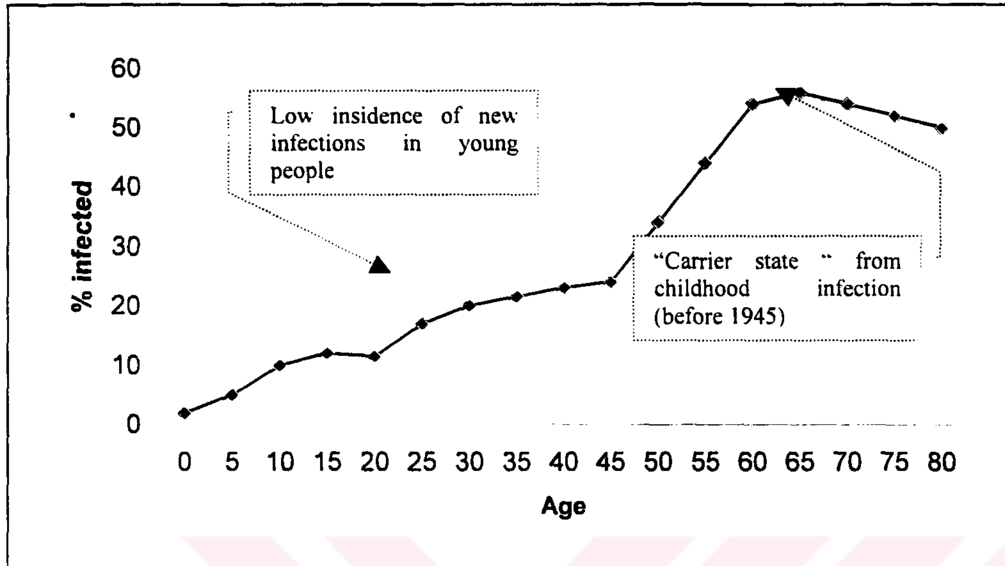


Figure 1 Epidemiology of H. Pylori in western countries (15)

Epidemiological studies confirm that the decline in most western countries coincides with the decreasing prevalence of Helicobacter pylori colonization as the result of better sanitary conditions and the widespread use of antibiotics (10).The majority of infected individuals are asymptomatic, but might have histological changes in the gastric mucosa (10).

It was suggested that most infections are acquired during childhood and carried in for the rest of the life (4). For instance, 70-years-old Americans carry H. pylori that they acquired during childhood when the contamination rate of their food and water were supposed to be higher than those at the present time. It was shown that less number of Americans born after 1950 carry the bacteria because of better life conditions (10). So it appears that the lower the standard of living, the higher the possibility of getting infected with Helicobacter pylori. Infection with H. pylori is more common in developing countries than in developed countries, which supports the above-mentioned findings (16). Most of the patients infected by H. pylori have low socioeconomic status. Roughly, this infection is associated with poor hygiene, crowded living conditions, sharing of beds during childhood. and a poor domestic water supply (16). In Peru, the water supply was incriminated to be the source of H. pylori infection (15). As seen in figure 2 most individuals from

Brazil, Africa, Asia and Eastern Europe were infected during their teen ages. It appears that 90% of individuals over 20 years of age were infected with H. pylori (15).

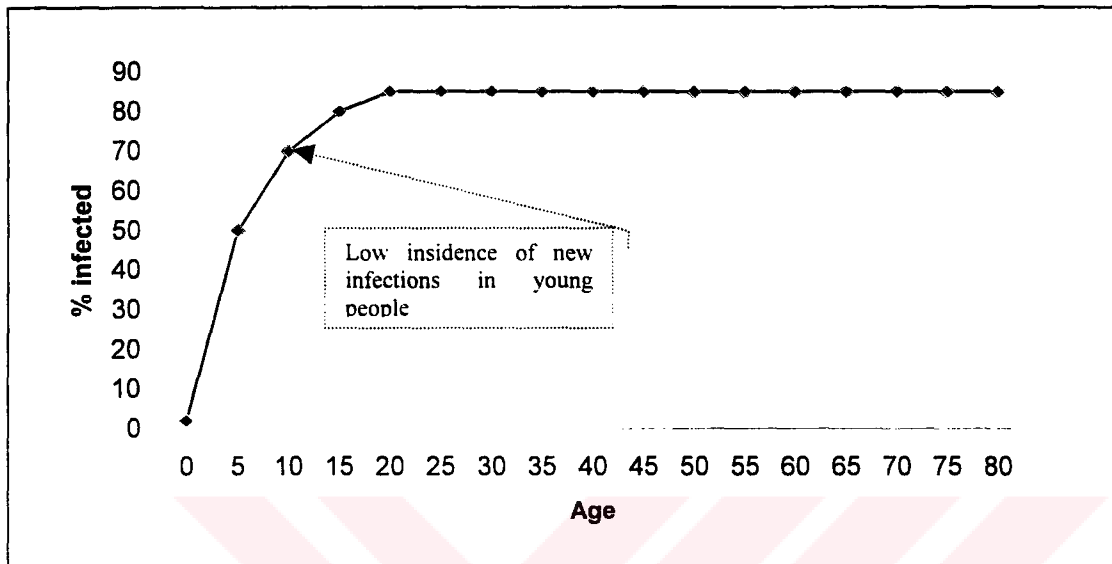


Figure 2 Epidemiology of H. Pylori in developing countries. (15)

The mode of transmission is most likely through the fecal–oral and oral–oral route that might occur during childhood (16). Another possible mode of transmission is iatrogenic in which H. pylori is introduced into another individual by contaminated endoscopy tubes (4,15).

Us and Hascelik (1998) studied a total of 657 serum samples collected from 331 male and 326 female who had no gastrointestinal complaints. Three hundred and forty eight subjects (53%) were seropositive for H. pylori by the ELISA test. The overall seropositivity rates did not differ with sex whereas antibody prevalence increased progressively with age. Their results showed that more than 30% of the subjects acquired infection before teenage and that about 70% of adults had antibodies to H. pylori in Turkish population (39).

3. Pathogenesis

The pathogenicity of bacterial infection in general has been associated with essential factors: attachment, toxicity, and invasiveness (25). Attachment is considered the first step in the establishment of pathogenicity. H. pylori possess

several virulence factors, many of these were known to play an important role in pathogenesis (table 1) (16).

• H. pylori virulence factors	• Mode of action
• Flagella	• Efficient motility in mucus.
• Specific attachment to phosphatidylethanolamine, GM3 ganglioside, Lewis B antigens	• Selective colonization of gastric, mucus-secreting, epithelial cells.
• Urease	• Survival in gastric environment.
• Catalase	• Survival in gastric mucosa and possibly within phagocytic vacuole (protection from H_2O_2) & resistance to killing by phagocytes.
• Heat shock protein A (HspA)	• Molecular chaperonins, Ni^2 uptake (HspA).
• Phospholipase	• Digestion of epithelial cell membranes and mucus layer. Increased wettability of the mucus.
• Protease	• Digestion of epithelial cell membranes and mucus layer. Increased solubility of mucus.
• NixA	• Ni^{2+} uptake for urease function
• Superoxide dismutase	• Resistance to killing by phagocytes.
• VacA	• Cytotoxic for gastric epithelium.
• IceA	• Unknown.
• CagA	• Unknown.
• Low molecular weight chemoattractant proteins	• Attraction of neutrophils and mononuclear cells that subsequently release reactive oxygen species and interleukins.

Table 1 Virulence factors of H. pylori and their mode of action (4,15,26)

a. Motility

H. pylori motility is achieved by the spiral shape, unipolar flagellae (15,16). It has been suggested that these flagellae may play an important part as pH sensors for the organism within the variably acidic environment of the gastric lumen and mucus layer (16).

b. Urease

The urease activity of H. pylori is higher than any other known bacteria and was found to be essential for survival of the organism in the highly acidic environment of the stomach, since urease deficient mutants lack the ability to colonize the stomach (16). The enzyme breaks down urea in the gastric juice, resulting in the generation of bicarbonate and ammonium ion around the bacteria that allow its safe passage through the gastric acid barrier and its arrival at the protective mucus layer (15). Ammonia elevates the pH of the gastric mucus layer from about 6 to 7. It was reported that increased gastrin RNA message in rat mucosa exposed to ammonia and agents that induce inflammation, suggesting that the hypergastrinemia present in patients with H pylori might be secondary to the presence of ammonia (H. pylori urease)., and H. pylori urease is increased in duodenal ulcer patients, because H. pylori density is greater in this group (15).

c. Catalase and Superoxide Dismutase

The genes encoding the catalase and superoxide dismutase enzymes of H. pylori, demonstrate significant homology to those of intracellular pathogenic microorganisms, suggesting a role in resistance to killing by polymorphonuclear leukocytes (4). A significant fraction of catalase and superoxide dismutase is associated with the surface of viable H. pylori; whether surface association of these enzymes is essential for protection against oxygen-dependent killing of H. pylori by neutrophils is not known (4).

d. Heat Shock Protein (Hsp)

The sequence of the gene encoding the heat shock protein B (HspB) of H. pylori is highly conserved compared to those of other bacteria and humans (4). This conservation suggests that the sequence cannot be modified without affecting the function of the protein. Based on structural similarity, HspB may function as a molecular chaperone for urease (4). The heat shock protein B gene (hspB) is par t of a bicistronic operon (hspA-hspB), which has been cloned and sequenced (4). The H. pylori hspA gene is unique in that it contains a nickel-binding site at its C terminus. Expression of the hspA and hspB proteins together with the H. pylori urease increases the activity of urease in functional complementation experiments.

Thus, hspA may play a role in the integration of nickel into the functional urease molecule (4).

e. Phospholipase

Once within the gastric mucus, H. pylori is able to attach to phspholipids, such as phosphatidylethanolamine, sialylated glycoproteins, such as ganglioside GM3, and Lewis B antigens present in persons with blood group O (16). Once attached to the mucus layer and mucosa, H. pylori delivers soluble proteases and phospholipase, which may be involved in the breakdown of gastric mucus and the phospholipids of the surfactant layer overlying the gastric epithelial cells. These include phospholipase A2 and phospholipase C. Moreover, the effects of acid and alkaline phosphotasess lead to further breakdown products of this process, including diglycerides, cholinephosphate and lysolecithin, which may form an important energy source for the bacteria (16).

f. CagA

CagA protein is another putative virulence factor of H. pylori that is encoded by the cagA gene (cytotoxin associated gene A) (17, 37).The strains carrying this gene enhance expression and production of IL-8 (40). It has been reported that this gene is present in about 60% of H. pylori isolates (17, 37). The presence of the cagA gene was correlated with the expression of CagA protein (46,53) In all Chinese isolates nearly all cagA gene positive strains express the protein (31). The cagA gene is positioned at one end of a pathogenicity island called cag pathogenicity island (cag PAI) with 30 other genes (17, 38). The primary sequence of the cagA gene contains repeat sequences in the 3' region whereas the structure of cagA reveals a 5' highly conserved region (41). The CagA protein has a variable molecular weight of about 120-168 kDa and initiates a marked immunological response (16). Variation in the size of the protein has been correlated with the presence of a variable number of repeat sequences located in the 3' region of the gene (41). Because CagA is strongly immunogenic outer membrane protein it is possible that repeats affect the host immune response. It has been suggested that the presence of repeat regions in the 3' region of the cagA gene may results with different immunogenecities (41).Yamaoka et al. identified four types of the cagA gene (types A to D) that differed in the structural

organization of their primary sequences, as a result of variation in the numbers of different repeat region (41). Type C strains has the longer cagA gene that results in CagA proteins of larger size. They illustrate that the primary gene structure of the 3' region of the cagA gene of H. pylori isolates in Japan showed that this region of cagA differs markedly from the primary sequence of cagA genes reported for Western isolates (41). Pan et al. indicated that the DNA sequences of cagA genes differs among the Dutch and Chinese H. pylori isolates (42). In Western populations, strains that contain cagA (indicating the presence of the pathogenicity island) are more strongly associated with more severe disease than strains that lack cagA. In Asian populations no significant association between cagA positivity and disease development was reported (43). Although the function of CagA is not known, the protein has been reported to be associated with particular gastroduodenal diseases (17). On the other hand CagA seroprevalence varies geographically too. In Western countries, CagA seropositivity has been shown to be higher in patients with atrophic gastritis, which is considered as a precursor to gastric cancer(44). However in Japan, high CagA seropositivity rates have been observed in asymptomatic adults. (44)

The function of CagA protein is not very well understood. CagA has been thought to be associated with IL-8 induction in the gastric epithelium (6,30), while bacterial eradication reduces IL-8 expression and neutrophil infiltration, the CagA protein may not directly cause IL-8 induction but indirectly by other proteins encoded by genes in the pathogenicity island upstream of the cagA gene (6). This is according to the recent in vitro studies that indicated that isogenic cagA mutants with disruption of CagA expression (deletion of cagA) also induce IL-8 production (17,30,40) and the deletion of the other genes in the cag PAI abolishes the ability of the bacterium to stimulate IL-8 production (17). These proteins may be coexpressed with CagA and thought to function as a secretion system for the export of bacterial factors in host epithelial activation (6,17,30,40). Virulent strains of the organisms frequently express the CagA protein, which has been reported to be an important marker of peptic ulcer disease. Therefore the CagA protein may be used as a phenotypic marker for virulence strains (17,6,30,40) and that the epithelial chemokine response appears to be dependent on the other multiple

genes present in the cag PAI (17). The cagA gene has also been isolated and is distinct from the vacA gene. More recently, two closely associated genes encoded on a defined region of PAI, initially named cagB and cagC have been identified and later renamed as picB and picC (16,30) according to their ability to promote the induction of cytokines (pic) in the host (16). Strains carrying the picB gene have also been reported to express the cagA gene in vivo. It therefore seems that cagA gene positive and picB gene strains have the ability to induce IL-8 release from gastric mucosa (30).

g. VacA

Helicobacter pylori produces cytotoxic proteins, the most important is the vacuolating cytotoxin (VacA) (15,16). The VacA protein has a molecular weight of about 87 kDa (45). The toxin cause vacuoles formation in isolated gastric epithelial cells and superficial mucosal ulceration in experimental animals and tissue culture cells (HeLa cells) (15,16,45,46). Although all strains of H. pylori possess the vacA gene, only about half the organisms secrete an active cytotoxin (4). Moreover, it has been suggested recently that there are genetic differences between the vacA allele of organisms. which secrete and do not secrete the toxin (4). This cytotoxin induces acidic vacuoles in the cytoplasm of eukaryotic cells; these vacuoles accumulate neutral red dye, facilitating their analysis. (4) The vacuolating activity of the cytotoxin is neutralized by specific rabbit antiserum; such neutralizing antibodies are also detectable in sera of many *H. pylori*-infected individuals including those who develop gastric carcinoma (4). On the other hand, the vacuolating assay and analysis of VacA protein expression produced slightly different results. The difference between vacuolating activity and expression of VacA protein suggests that some strains secreted either inactive cytotoxin or only a small amount of active cytotoxin, so that the cytotoxin was detected by immunoblot analysis but its activity was not detected by the vacuolating assay. Indeed, some strains such as Tx30a secrete inactive cytotoxin, so that no vacuolation was observed by the vacuolating assay while VacA protein was detected by immunoblot assay (17).

The cloning and sequencing of the vacA gene (3.9 kb in size), which encodes the vacuolating cytotoxin was reported earlier (4). The vacA gene

encodes a 139 kDa protoxin that contains a leader sequence of 33 amino acids, the cytotoxin itself (VacA) and a C-terminal fragment of approximately 50 kDa that exhibits homology to a C-terminal fragment of the IgA protease precursor of Neisseria gonorrhoeae (4). The C-terminal fragment of N. gonorrhoeae IgA protease is known to be involved in the translocation of the protease through the outer membrane. By analogy, the C-terminal fragment of the H. pylori protoxin may play a role in the secretion of cytotoxin (4). Isogenic H. pylori mutants with mutations in the vacA gene have been constructed. As expected, these mutants do not express the cytotoxin and lack vacuolating activity. The cytotoxin does not exhibit significant relatedness to other known toxins that might give clues to its mechanism of action. Cytotoxin-induced vacuoles appear to be derived from the late endosomal compartments within eukaryotic cells (4).

Furthermore, Helicobacter pylori vacA genes exhibit allelic mosaicism such that each gene has one of two different signal sequences (s1 and s2) and also one of two alternative sequences in the middle region of the gene (m1 and m2). The vacA signal sequence type s1, but not s2, was closely associated with in vitro cytotoxic activity, PUD, and the presence of the cagA gene (17,46). m1 strains are associated with increased gastric epithelial damage (17). The presence of cagA correlates with the presence of the s1 m1 vacA genotype; while the absence of cagA correlates with the s2 m2 vacA genotype (28,47). The significance of this relationship is that s1 m1 VacA is highly active (47). Serum IgG anti-VacA antibodies were present more frequently in patients carrying type s1/m1 strains than in other H. pylori-positive patients. In addition, the highest levels of IgA anti-VacA antibodies were detected in the gastric juice of patients carrying type s1/m1 strains (51). As a result, different VacA isoforms have distinct antigenic properties and that multiple forms of VacA elicit antibody responses in H. pylori-positive humans (51). There seems to be no functional link between cagA gene and VacA protein (47). Maeda et al. indicated that CagA protein is strongly associated with vacuolating cytotoxin activity in Japan (6).

h. IceA

The recently discovered iceA gene (induced by contact with the epithelium) , a locus that has two major variants, iceA1 and iceA2, among diverse

H. pylori strains. IceA1 is upregulated upon contact of H. pylori with the gastric epithelium (4,26). Studies indicated that infection with an iceA1 strain is strongly associated with duodenal ulceration and a marker for peptic ulcer disease (4,26,32).

i. Pathogenesis

Following infection, H. pylori colonizes predominantly the antral part of the stomach, which is facilitated by its powerful urease activity and its motility, that is aided by the spiral structure of the organism and the multiple flagellae. H. pylori urease is 100 times more potent than the other bacterial ureases and hydrolyses urea to ammonia and also assists H. pylori in exporting intracellular hydrogen ions to form ammonium. While this creates an alkaline microenvironment around the organism it also damages the surface of the gastric epithelial cells. Urease, together with a spectrum of bacterial products, such as protease, catalase, leads to weakening of the mucous bicarbonate layer and damage to surface epithelial cells.(14). The most important factor dictating the development of peptic ulcer appears to be whether the H. pylori organism produces soluble cytotoxins that cause vacuolation of the epithelial cells and serve to stimulate the production of interleukin-8 in the mucosa, which in turn leads to more marked attraction of polymorphonuclear leukocytes. Individuals who harbour toxin-producing H. pylori have, on average, greater numbers of organisms in the mucosa and more acute inflammation reaction than the actual H. pylori organism (10). As mentioned before colonization by H. pylori also leads to the release of a repertoire of cytokines associated with a marked mucosal inflammatory response, which results in further damage to the mucosa and disturbs the regulatory mechanisms of gastric acid secretion by inhibiting the release of somatostatin and gastrin (14). Individuals with gastritis have diminished numbers of D cells and lower somatostatin levels as a result. Since somatostatin acts locally to suppress antral G-cell gastrin secretion, patients tend to have higher gastrin levels, greater basal acid secretion, and hypertrophy of the gastric corpus (acid-secreting) mucosa. So H. pylori appears to cause an acid hypersecretion seen in patients with duodenal ulcer. In persons who do not develop peptic ulcer, lifelong H. pylori gastritis can lead to intestinal

metaplasia (replacement of gastric mucus epithelium with intestinal brush-border and goblet cell-type epithelium) (10).

C) The presence of CagA & VacA genes and their association with peptic ulcer diseases

Helicobacter pylori infection is strongly associated with the pathogenesis of a variety of gastroduodenal diseases, such as chronic active gastritis, gastric ulcer, duodenal ulcer, gastric atrophy, intestinal metaplasia, lymphoreticular hyperplasia, mucosal-associated lymphoid tissue (MALT) lymphoma and adenocarcinoma (2). Although chronic active gastritis appears in all infected individuals, only a small proportion will develop peptic ulcer disease (2).

Xiang et al. (1995) have divided H. pylori into two types, type I and type II, according to the presence or absence of two antigens, CagA and VacA. Type I strain possess both cagA and vacA genes, which codes for the production of CagA and VacA proteins while Type II strains does not produce such proteins (33).

It was reported that the Type I strains were strongly associated with peptic ulcer disease in the host (6,17,45). Maeda et. al. (1998) concluded that most H. pylori strains in Japan produce both VacA and CagA proteins not only in vivo but also in vitro. The isolates were positive for the vacuolating assay and CagA protein in 79% of the individuals examined (6). Weel et. al. (1996) reported that infection with H. pylori Type I strains was detected in 43 out of 76 (56.6%) patients with peptic ulcer, but in only 28 out of 76 (36.8%) patients with non-ulcer dyspepsia in the Netherlands. An association between type I H. pylori and PUD was suggested. (45). In contrast, to the study done in Japan the prevalence of infection with Type I strains was found to be 25/33 (76%) in patients with peptic ulcer and 18/25 (72%) in those with non-ulcer dyspepsia (17).

Shimoyama et al. (1999) reported that VacA seropositivity was 68% in patients with gastric cancer and 70% control subjects. They suggested that VacA seropositivity is not associated with increasing risk of gastric cancer in Japanese

populations (48). In their earlier report (1998) they showed that CagA seropositivity was 60% in cancer patients and 44% in control subjects , a fact that indicates the association of CagA seropositivity with increased risk of gastric cancer in Japanese population (49). Maeda et al. (1998) on the other hand did not find any significant correlation between the expression of CagA and VacA proteins by H. pylori isolates and gastroduodenal status of the host in their study that is done in Japan and published during the same year. They suggested that these virulence factors cannot be used as markers to discern the risk of developing serious gastroduodenal disease in the host (6). It was reported that the CagA antibody seropositivities of asymptomatic controls (81.8%) and patients with nodular gastritis, gastric ulcers, and duodenal ulcers (80.0-95.0%) were not significantly different in Japanese patients (44). Rudi et al. (1997) reported a twofold increased risk of gastric cancer in patients with antibodies against VacA and CagA to be significantly associated German population. Their results were in agreement with more recently findings on the association between CagA status and cancer risk (50).

Sozzi et al. (1998) indicated that infection with CagA-positive H. pylori strains is associated with an increased prevalence and intensity of antral atrophy and intestinal metaplasia, in addition to higher degrees of gastritis. They suggested that the CagA status could be a helpful parameter to define a subgroup of H. pylori-infected patients at increased risk of developing gastric adenocarcinoma (37). The presence of the cagA gene and the expression of the CagA protein are found more often in patients with gastric atrophy than in those with chronic superficial gastritis alone (16). Vorobjova et al. (1998) detected anti-CagA IgG antibodies in 63% of the Estonian population and in 87% of patients with gastric cancer (52). Takata et al. (1998) reported a significantly higher CagA positive isolates in both duodenal ulcer (97,6%) and gastric ulcer (83,9%) patients than in the non-ulcer dyspepsia patients (61,4%). They indicated that CagA-positive phenotype may be important as a virulence marker for peptic ulcer disease independent of the presence of VacA, vacuolating cytotoxin activities (54). Torres et al. (1998) studied the prevalence of anti-CagA antibodies in Mexico and reported that individuals seropositive for CagA, but seronegative for H. pylori

whole cell antigen, were more frequent in areas with higher gastric cancer rates. Their results supported the possible role of CagA(+) status as predictor of risk for gastric adenocarcinoma in Mexico (5).

D) The inflammatory and immune responses to H. pylori

According to one of the scenario regarding inflammatory responses to Helicobacter pylori, the initial response to H. pylori appears to have a marked neutrophilic component. It is thought that both bacterial factors and induction of interleukin-8 (IL-8) result in this initial inflammatory response (14). Some of the products of Helicobacter pylori induce the adhesion of neutrophils to endothelial cells and neutrophil/ monocyte chemotaxis and activation. These upregulate the expression of CD11b/CD18, which facilitates ICAM-1 (intercellular adhesion molecule-1) neutrophil adhesion to endothelium and tissue extravasation (Figure 3) (14). Neutrophilic responses also indirectly result from host derived chemotactic cytokines, bioactive lipids such as LTB_4 and the product of antigen-specific IgG responses in the gastric mucosa. The gastric epithelium like the other epithelia at the other mucosal sites, primarily block the bacteria and the gastric epithelium is known as one of the major source of IL-8, which possess potent chemotactic activity for neutrophils and T lymphocytes (14).

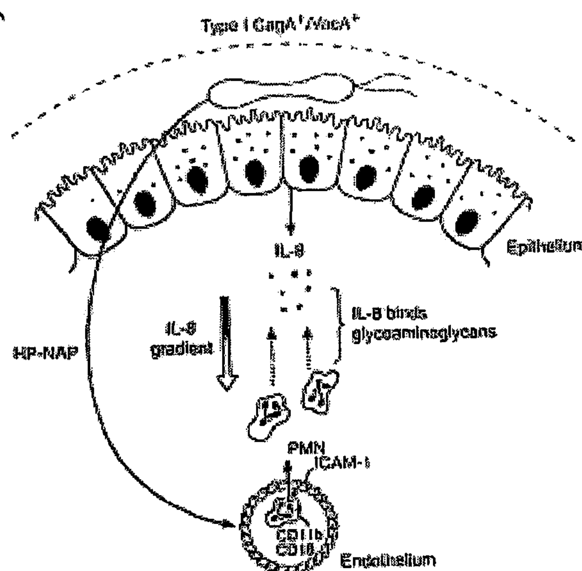


Figure 3. **Pathways of neutrophil (PMN) stimulation following initial Helicobacter pylori infection. CagA, cytotoxin-associated protein; VacA, vacuolating toxin; IL, interleukin; HP-NAP, H. Pylori neutrophil activating protein; ICAM-1, intercellular adhesion molecule-1. (14)**

It has been observed in an in vitro study that once H. pylori adhere to the gastric epithelial cells, the bacteria induces a wide range of intracellular changes such as increased expression of the gene coding for IL-8 and resulting in increased secretion of this cytokines. This shows that gastric epithelial cells take an essential role in the regulation of mucosal cellular responses to pathogens (14). Though IL-8 activity is weak in the epithelium of uninfected ones, it is significantly increased in the epithelium of chronic gastritis patients together with increased mucosal production of other cytokines. Tumor necrosis factors (TNF) and IL- 1 following activation of transcription factors upregulate IL-8 expression (14). Both of these cytokines are seen locally during chronic gastritis. During acute infection, two ones; neutrophil infiltration and epithelial damage by the vacuolating toxins facilitate mucosal uptake of bacterial products and induction of cytokine secretion from mucosal macrophages so all these result in amplifying IL-8 expression in the epithelium. In addition neutrophils produce inflammatory cytokines such as IL-1, IL-8 and TNF-α. This also causes the amplification of cellular responses to infection (14) as shown in Fig. 4.

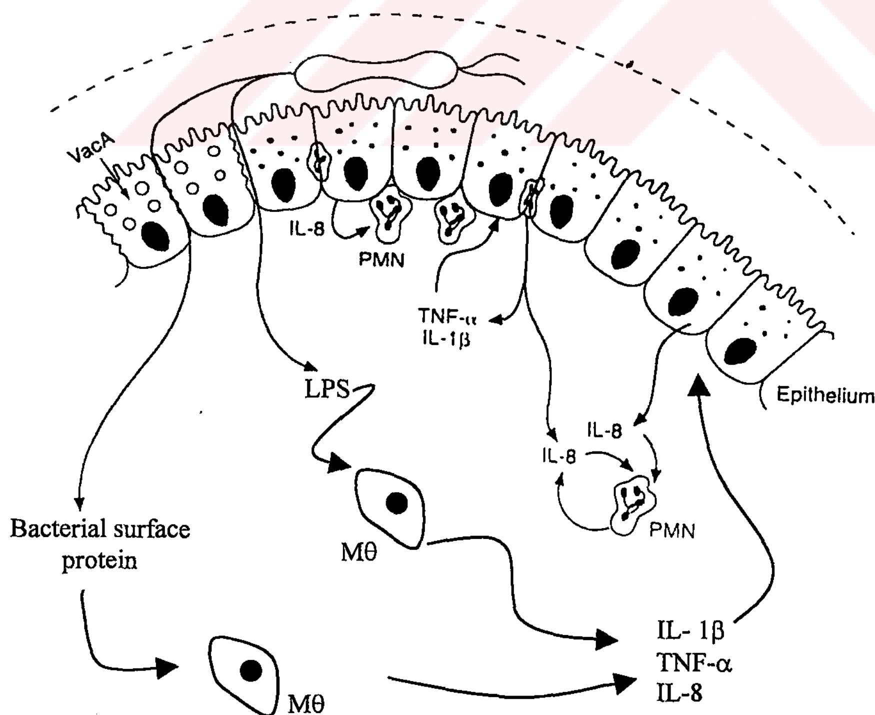


Figure 4 Pathways of Helicobacter pylori induction of epithelial and mucosal interleukin (IL-8). VacA, vacuolating toxin; LPS, lipopolysaccharide; PMN, neutrophil; TNF-α, tumour necrosis factor-α; Mθ, macrophage. (14)

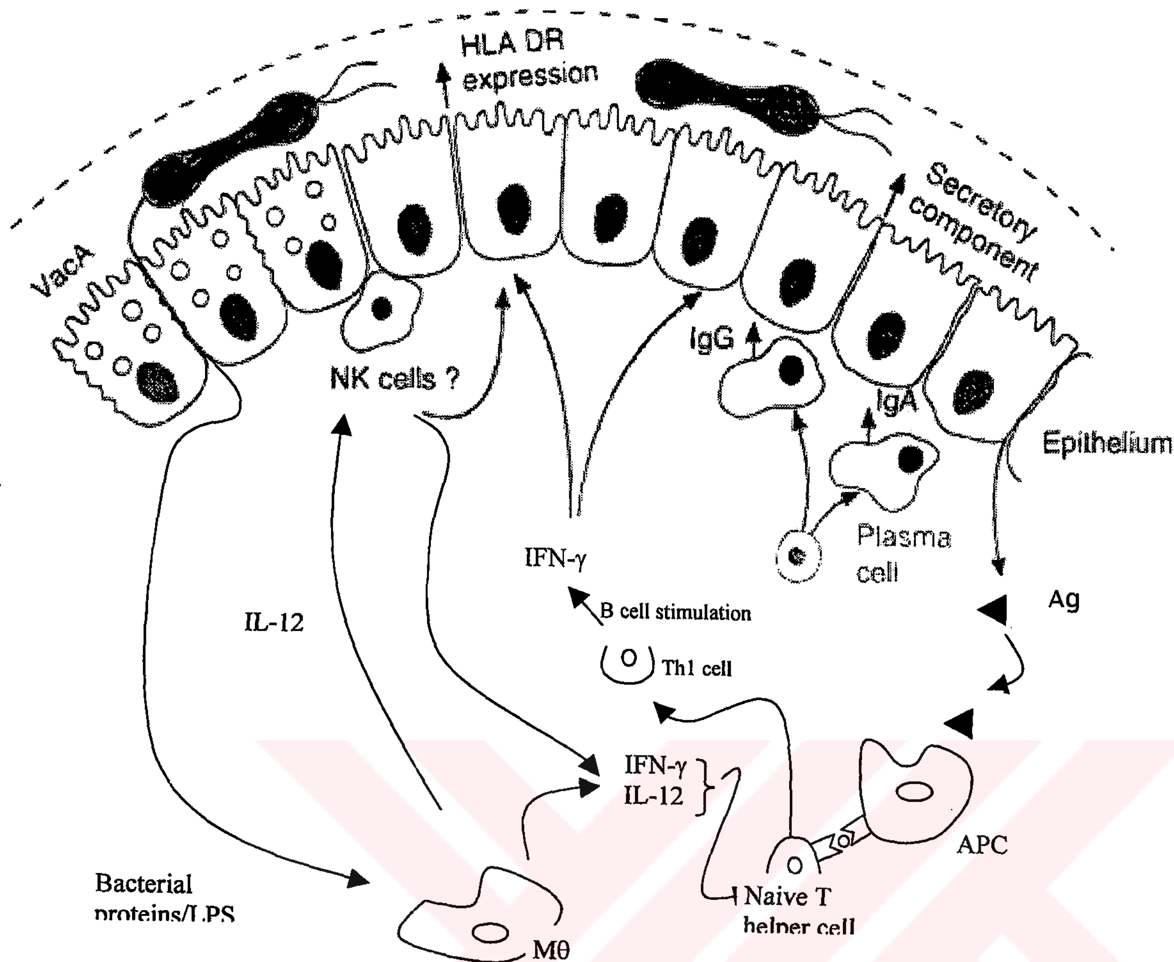


Figure 5. Induction of antigen-specific responses to Helicobacter pylori in the gastric mucosa. VacA, vacuolating toxin; HLA DR, homan leukocyte antigen DR; NK, natural killer; IFN-γ, interleukon-γ; IL, interleukin; LPS, lipopolysaccharide; Mθ, macrophage

Interferon-γ secretion and non-major histocompatability complex (MHC)-restricted natural killer (NK) cell activity are induced by interactions between peripheral blood lymphocytes and H. pylori (14). They play a very important role in antigen-independent activation of phagocytoses and in promoting expansion of antigen-specific T cell responses of the T–helper 1 (Th1) type. Neutrophils and monocytes, which are stimulated by bacterial infection, produce IL-12, which is a potent stimulator of NK activity (14). According to the animal studies interferon-γ and IL-12 are considered to be critical in driving Th1 development and cell-mediated responses to infectious agents. Current evidence suggests that the gastric mucosal T cell response to H. pylori is predominantly of the Th1 type (14) as shown in Figure 5.

It was shown that natural phenotypic variants, with disparate cytotoxin and CagA expression, show that induction of IL-8 secretion does not relate to bacterial cytotoxicity (14). In spite of the association between the induction of IL-8 and CagA expression, studies with a cagA isogenic mutant strain demonstrated that CagA protein is not a direct inducer of IL-8 and the products of genes co-expressed with CagA in type I strains are necessary for the induction of this inflammatory epithelial cytokine response (14).

It can be said that the CagA is a very immunogenic phenotypic marker for H. pylori strains causing pro-inflammatory epithelial responses. Recent studies have shown that mucosal IgA recognition of the CagA protein is strongly associated with epithelial IL-8 expression (14,29,30). H. pylori eradication in patients suffering from duodenal ulcers causes a marked decrease in both IL-8 and TNF-α mRNA expression in the antral mucosa (14). Yamaoka et al. found that IL-8 mRNA expression was significantly more common in gastric mucosal biopsy specimens from cagA gene positive gastritis than those from cagA gene negative gastritis using reverse transcriptase RT-PCR (30). Whereas they demonstrated the mucosal levels of IL-1β, IL-6, IL-8, and TNF-α were significantly higher in H. pylori positive than in H. pylori negative specimens, the mucosal levels of IL-1β and IL-8 were significantly higher in $cagA^{+}$ than $cagA^{-}$ strains (14).

An association was found between pepsinogen C, which is produced by the body cells, fundus chief cells and the mucus neck cells of pyloric glands in the antrum and cagA (28). It was hypothesized that cagA positive strains might give rise to an increased production of mucosal cytokines because they cause a higher degree of mucosal inflammation. Cytokines, in turn, may influence the production of anti-H. pylori antibodies (28).

Further evidence supporting strain variation in the generation of inflammatory cellular responses comes from the studies of Rautelin et al. About one-third of 61 strains of H. pylori were found to induce a strong rapid oxidative burst in neutrophils in the absence of opsonins. Such strains were more common in patients with peptic ulcers than in those with only chronic gastritis (14).

T.C. YÜKSEKÖ

It is now clear from in vitro studies that the ability to induce innate immune responses and cellular activation varies with different H. pylori strains. Recent studies in murine models of H. pylori infection also strongly support the concept that type I induce a great degree of gastric inflammation than type II strains (14).

Chronic active gastritis

Following the stimulation of innate defence mechanisms, continued exposure of the gastric mucosa to H. pylori antigens results in the generation of a specific immune response. Such gastric responses to a non-invasive organism, in the absence of Payer's patches and M cells, probably arise following uptake of shed bacterial products, direct epithelial endocytosis of bacterial antigens or passage of such material through disrupted tight junctions of the gastric epithelium (16). Antigen uptake in the intestine during transit of released bacterial antigens and/or bacteria is also feasible. The marked degenerative changes evident in the gastric epithelium during acute infection will facilitate antigen uptake and the development of specific responses in newly infected subjects. Gastric mucosal absorption of the urease enzyme has been demonstrated immunohistologically and virtually all patients with chronic gastritis have a specific gastric mucosal IgA response to H. pylori (16). This response is evident even in the few H. pylori negative patients with inactive chronic gastritis, suggesting previous recent exposure to bacterial antigens. IgM responses are generally only found locally in the acute stage of infection. IgG plasma cells, particularly IgG1, are increased in gastritis and a H. pylori-specific IgG response has been demonstrated locally in the gastroduodenal mucosa (16).

Following the establishment of chronic gastritis, a systemic humoral response develops, with virtually all infected patients having serum IgG antibodies to the bacterium (16). A systemic IgA response to H. pylori is also found in some individuals, but IgA antibody titers are generally considered less useful in the diagnosis of infection. As with the mucosal IgA response, there is a marked heterogeneity in the specific antigens recognized by the systemic IgG response.

H. pylori- specific IgG titers decline after bacterial eradication and serological tests can be useful in monitoring the success of eradication therapy (16).

The importance of the IgA and IgG mucosal antibody response to H. pylori in the regulation of infection and the immunopathology of gastritis is unclear. The presence of secretory IgA at mucosal surfaces is likely to be important in inhibiting antigen uptake, blocking bacterial adherence and motility, and toxin neutralization. Gastric IgA antibodies have been shown to inhibit the vacuolization of epithelial cells induced by the cytotoxin in vitro, suggesting that a protective immune response might exist in vivo. As IgA antibodies do not efficiently activate complement, this isotype may also have a functional role in blocking H. pylori specific IgG-mediated complement activation and associated release of neutrophil-activating inflammatory mediators (16).

IgE plasma cells are also present in the gastroduodenal mucosa, particularly in regions of ulceration. IgE-mediated responses may, therefore, be important in the pathogenesis of mucosal damage. H. pylori specific IgE antibodies are present in the serum of infected subjects, and H. pylori antigens will induce histamine release from basophiles of infected subjects in vitro (16).

H. pylori-specific T cells are present in the gastric mucosa of infected patients. Studies of mucosal T cell populations isolated from patients with chronic gastritis have demonstrated an increase in cells secreting interferon-γ. The local secretion of interferon-γ is important in up regulating gastric epithelial expression of class II MHC molecules, a situation that occurs in chronic gastritis (16). Interferon-γ is also likely to play a functional role in increasing epithelial expression of a secretory component, the specific receptor for J-chain-containing polymeric IgA and IgM immunoglobulins. Antigen-specific T cell responses will have an important role, therefore, not only in regulating humoral responses to H. pylori but also in modulating epithelial gene expression of key molecules for mucosal defence and antigen presentation (16).

E) Diagnosis of Helicobacter pylori infection

The sensitivity of any diagnostic test can be defined as its ability to give positive results for all patients infected with the bacterium, while the specificity of a test is its ability to give a negative results for all those not infected. These definitions point to the need for a reference method or 'gold standard' that is able to determine all those truly infected. Unfortunately, no single technique currently used can fulfil this criterion. A solution is to use the results of a combination of two or more techniques that are considered to be reliable, and compare them with the results of the technique being evaluated. A true-positive result can be defined as a case where two or more techniques are positive, and a true-negative result is where all of the tests are negative (20).

The sensitivity of a test may vary depending on whether it is performed before treatment or 4–6 weeks after treatment to eradicate the bacterium. In the latter case, even if the bacteria are still present, the concentration is likely to be much lower because the balance between the aggressor (H. pylori) and the host's defence has been tilted in favor of the host's defences. Low numbers of the bacterium will alter the results of sensitivity and specificity (20).

Several tests have been employed for the detection of Helicobacter pylori infection. These are classified into;

a. Biopsy-based tests (invasive tests) that detect the bacteria in biopsies obtained during endoscopy;

1. CLO-test
2. Culture
3. Histology
4. PCR

b. Non biopsy-based tests (non-invasive tests) that requires no endoscopy and detects the presence of bacteria indirectly;

1. Urea Breath Test
2. Serology

Comparison of the above mentioned diagnostic tests are shown in table 2.

Table 2 Comparison of the diagnostic tests currently available for the detection of H. pylori

Test	Sensitivity (%)	Specificity (%)	Endoscopy required	Comments
CLO	89-98	93-98	Yes	Endoscopic method of choice for diagnosis of H. pylori infection
Culture	75-95	100	Yes	For susceptibility testing and detailed characterization of isolates
Histology	93-98	95-98	Yes	Multiple antral biopsy specimens recommended; special stains improve sensitivity
PCR	85-96	90-100	Yes	Assess DNA, not necessary viable bacteria; useful to assess H. pylori strain differences including antibiotic resistance
^{13}C-UBT	90-95	90-95	No	Assesses existing load of variable bacteria well studied to follow-up of antimicrobial therapy.
^{14}C-UBT	90-95	90-95	No	As above for ^{13}C-UBT; low radiation exposure
Serology	88-95	86-95	No	Not appropriate for short-term follow-up of antimicrobial therapy; excellent epidemiologic tool

a. Biopsy-Based Tests (Invasive Tests)

1. CLO-test

Most research has been performed with commercially available tests. Hp fast® uses an indicator providing a gradient of colors that may help to quantity the number of organisms present. In the Camplyobacter-like organism test (CLO) test® that was developed by Marshall and the first of the commercially available biopsy based urease tests designed specifically for H. pylori detection (4) and the HUT® test, which use phenol red as a pH indicator, the delay before the color changes may give an indication of the quantity of bacteria present (21).

CLO test is particularly suited for the detection of H. pylori. The sensitivity and specificity are dependent on the time lapse before the results are read. Within 1 hour or less, the specificity is very good but the sensitivity is not optimal, it will increase after 24 hours, but the specificity decreases because contaminant bacteria harboring urease can give a false-positive result (20). A false-positive result can also be seen when tracers of blood are interpreted as a red stain, which occur in 2-5% of the cases (20).

The sensitivity of CLO test depends upon the number of bacteria present in the biopsy specimen. A 10^4 organisms are required for a positive result (20). Hence ,the test is unsuitable for determining the success of H. pylori eradication treatment as the number of the organisms would not have usually reached the critical level 4 weeks after treatment, in the case of failure. The sensitivity of the test before treatment is in the range of 75- 85%, when reading is performed within 1 hour. This level of sensitivity is satisfactory when testing patients for inclusion in explicative clinical trials, but may be a problem in pragmatic trials as 10-20% of patients who are infected, but with a low number of bacteria, are not included (20). Thus it should be used as the sole method of detection of infection in ordinary practice. In the event of a negative result, another test should be used. Increased sensitivity may be achieved by using two biopsies, by incubating the test at 37°C and by using unbuffered media. The latter method is not recommended, however, because it can produce false-positive results (20).

2. Culture

Culture of H. pylori has two major advantages. First, it allows antimicrobial susceptibility testing; second, isolates obtained by culture can be characterized in detail (4). In theory, culture is the most sensitive technique because even one organism in the specimen will multiply to produce a population of billions (i.e. a colony) and thus give a positive result. Unfortunately, in the case of H. Pylori, bacteria seen on a smear may not always grow on the plate because of problems with the conditions in which they are grown in the laboratory. During transportation, it is essential to maintain the specimen at 4°C, and to avoid desiccation and contact between the specimen and air. When such conditions are fulfilled, biopsies can be plated within 24 hours. An alternative is to freeze the biopsies at –70°C or in liquid nitrogen and to thaw them just before inoculation of the culture (20).

For growth of the cultures, it is essential to use at least two different media (selective and non-selective) that have been freshly prepared, to grind the biopsy and to incubate at 37°C in a microaerobic atmosphere, with humidity, for at least 10 days. The specificity of culture is undoubtedly the best of all of the

techniques because, once colonies are established, it is possible to perform all the tests required for the identification of the bacterium (4).

3. Histology

H. pylori can be visualized in conventionally stained sections with hematoxylin and eosin (H & E). Bacteria are seen located in the mucus adherent to the surface epithelium and are often found deep within crypts (4). The use of Giemsa stain improves the detection and visualization of the organism. The Sydney system proposes that two biopsies should be taken from each site within the stomach to avoid any sampling error. An experienced pathologist will, on the discovery of polymorphs, search further in cases where the biopsy initially indicates no H. pylori infection (4). The specificity of histological tests, like the sensitivity, depends on the expertise of the pathologist: it can be difficult to identify H. pylori when the few bacteria present are of atypical morphology. A possible aid is the use of immunohistochemistry (4).

A further limitation on the uses of histology, with regard to sensitivity and specificity, is the quality of the biopsies. If the biopsy is to small, poorly orientated, or inappropriately fixed or stained, the results will be affected; indeed, it may not be possible to carry out the test. The problems in using histology arise because the results depend on the observer; when the same specimens have been submitted to several pathologists, the concordance of results has been disappointing (20).

4. PCR

This new technique is reputed to be extremely sensitive and specific. Although, theoretically, only one copy of the target DNA is needed for detection in a specimen of water, in biological material the efficacy of amplification may be less and inhibitors of Taq polymerase may be present (21).

A potential advantage of PCR is that it may enable the diagnosis of H. pylori to be made non-invasively by detecting H. pylori DNA in non-gastric fluids such as saliva where 84% sensitivity was detected in one study, but many

investigators have not been able to routinely detect H. pylori DNA in saliva even among patients with proven gastric H. pylori infection (4).

Most results indicate that the sensitivity of PCR is, in fact, close to that of culture, but for variation of eradication, PCR may be slightly superior. One way to increase the sensitivity of the technique is to perform a nested PCR, that is, a second PCR on the amplified products with primers internal to the first sequence amplified (20). Because of a high risk of contamination linked with manipulation of amplified products, however, the use of this procedure should be discouraged. A second PCR should be performed using another set of primers and the biopsy considered positive when at lest one of the two PCR tests in positive. When PCR is performed on specimens other than gastric biopsies, the question of specificity is crucial, and only specimens showing positive results for both PCR tests should be considered as harboring H. pylori (20).

Claims have also been made that false positives can occur in biopsy specimens from endoscopic material that has not been properly washed, and it is, therefore, advisable to perform appropriate cleaning procedures to avoid contamination when PCR is performed (21).

b. Non-Biopsy-Based Tests (Non-invasive Tests)

1. Urea Breath Test

The principle of urea breath tests (UBTs) for the diagnosis of H. pylori is similar to that of other urease based tests. Urea is provided as a substrate which, in the case of the UBT, is ingested as either [^{13}C] or [^{14}C] urea. H. pylori urease hydrolyses the ingested urea into labeled bicarbonate, which is exhaled as labeled CO_2. The ^{13}C isotope is detected by mass spectrophotometer most commonly, although other analytical methods have been developed while the ^{14}C isotope is detected with a scintillation counter. Some microorganisms within the oropharynx also may hydrolyze urea; therefore, if urea is presented in liquid form, an early peak in labeled CO_2 may occur during the breath test. Thus, the timing of breath collection, the form of delivery of urea (liquid versus tablet form), the gastric emptying time, and other factors may influence the accuracy of the test. Overall, the performance characteristics of both ^{13}C and ^{14}C tests are similar (4).

The USA food and drug administration (FDA) has approved a commercial ^{13}C-UBT and it is possible that a ^{14}C test will be approved shortly. The importance of FDA approval for these tests should not be underestimated. Field trials of the now commercially available ^{13}C Meretek UBT led to changes in the cutoff value between positive and negative results(4). Other refinements in packaging and quality control are apparent. Although a single breath sample after ^{13}C urea ingestion is theoretically sufficiently discriminatory for defining the H. pylori status, two baseline breath samples are obtained. These paired duplicate samples should minimize inadmissible results ascribed to poor collection or inadequate transfer of breath samples to the tubes, faulty sealed, tubes that break and other factors. The accuracy of the test in the absence of the treatment with agents that suppress H. pylori has been approximately 95% in field trials (4).

A variety of ^{14}C protocols also have been used, but the Trimed test is the one most likely to gain FDA approval. In this protocol, the dose of ^{14}C is low, (1 μCi), the equivalent of less than 1 day background radiation . Advantages include the delivery of ^{14}C urea in the tablet form, thus avoiding the problem of detecting urea positive oral bacteria, no meal is given, minimal background levels of ^{14}C in contrast to that of ^{13}C and only a single breath sample need to be collected. Thus the sensitivity and specificity is excellent as that of the ^{13}C Meretek test, however the ^{14}C test is not recommended for use in children and pregnant women (4).

2. Serology

Infection of the gastric mucosa with H. pylori results in systemic as well as local immune responses, including elevation of specific IgG and IgA levels in serum and elevated levels of secretory IgA and IgM in the stomach, thus allowing the development of serologic tests for detection of the bacteria (4). Serologic methods have proven especially valuable in screening large numbers of individuals in epidemiologic studies. Such tests are noninvasive, relatively rapid and simple to perform, and much less expensive than tests requiring endoscopic biopsy. Further, serologic tests are less likely to be confounded by suppression of H. pylori infection by bismuth compounds, proton pump inhibitors, or antibiotics taken for unrelated conditions than are biopsy-based tests, which are dependent upon and reflect the current bacterial load (4). Although a wide variety of serologic

methods for detection of H. pylori have been described in the literature, most tests available commercially are enzyme-linked immunosorbent assay methods (4). The sensitivity of serology using ELISA is very good. In a study comparing this technique with biopsy-based tests, failure of a systemic response was noted in only 2% of the cases (20). In children, false negatives can occur up to a few weeks, or even months, after a new infection because the subject has yet to mount an immune response. Also, in some cases, because *H. pylon* infection is only a mucosal infection, antigenic stimulation can be low, emphasizing the importance of the cut-off value (20).

The utility of any serologic test for the detection of H. pylori-specific antibodies is dependent on the antigen preparation used. In general, three types of antigen have been used. These include crude antigens such as whole cells and whole-cell sonicates, cell fractions such as glycine-extracts and heat-stable antigens, and enriched antigens such as urease and a 120-kDa antigen (4). The sensitivities and specificities of tests involving all three types of antigen preparations typically approach 95%. However, recent meta-analysis studies of 11 commercial enzyme-linked immunosorbent assay kits and one latex agglutination kit found an average sensitivity of 85% and specificity of 79% (4).

In the absence of therapeutic intervention, antibody levels remain elevated, perhaps for a lifetime, reflecting the duration of infection. After eradication of H. pylori, specific IgG and IgA levels tend to decrease, typically to approximately half of the pretreatment value within 6 months (4,19). Low levels of specific IgG tend to persist for months even after eradication of H. pylori; therefore, using serologic tests to assess the effects of treatment may be problematic unless the pre- and post treatment sera can be directly compared (4).

There are three types of blood test available for the detection of H. pylori infection: those that detect an antibody response; tests of the pathophysiological state of the stomach; and those that indicate an active infection. The ELISA based kits are the most numerous of the commercially available tests. Originally the kits used crude antigen preparations but many of the newer kits use a more purified antigen preparation giving increased specificity but a lower sensitivity. The

combination of serology and serum concentrations of gastrin and pepsinogen may be used effectively to detect serious gastroduodenal disease in H. pylori infected patients (24).

Table 4 Comparison of commercially available ELISA kits for the detection of Helicobacter pylori infection (24).

Kit	*Sensitivity (%)*	*Specificity (%)*	*PPV (%)*	*NPV (%)*
Helori-test	98-99	88	94	88
Pyloristat	91-99	70-94	80	84
Pylori ELISA II	100	96	97	100
Helico G	71-97	65-95	89-90	65-98
Premier HP	85-100	80-100	76-100	88-100
Cobas Core	87-98	83-98	87	86
Pyloriset	81-97	69-97	76-97	51-98
Pyloriset update	100	79	95	100
Hel-p Test	89-100	62-93	65-90	91-1 60
Malakit	79-87	86-98	96	60
GAP IgG	76-100	26-99	76-100	71-100
HP kit Radim	81	90		
Roche MTP	94-98	83-86	86	90
HpG screen	83-93	68-91	66-84	84-100
Microstar	97	76	80	98
SIA Sigma	85-90	80-98	76-96	88-100
HM Sigma EIA	83-98	80-96	76	86
Autozyme	89	52	58	87
Pyloragen	79	75	71	83
Enzygnost	80	74	70	83
Quidel EIA	89	66	68	89
Enzwell	90	71	71	91
Color Vue	88	86	63	87

PPV, positive predictive value ; NPV, negative predictive value

There are several commercially available ELISA kits that vary in their antigen preparation and thus in sensitivity and specificity (24). The test can also be affected by the population under test and coexistent disease in the patients (24). Table 3 lists the main commercially by available serological assays including ELISA for the detection of H. pylori (24). The sensitivity, specificity, positive

predictive value, and negative predictive value at the above shown ELISA kits were given in table 4 (24).

III. MATERIALS AND METHODS

Patients

Forty-five patients attending the endoscopy unit at the Okmeydanı hospital mainly complaining of dyspepsia and abdominal pain were selected randomly. Twenty-six were males and nineteen were females of age range 16-80 years (mean 46). All patients were undergone endoscopy and three biopsies were taken from the antral part of the stomach (one for CLO, one for culture, one for histopathology). A serum samples was also obtained from each patients and stored at -20^0C.

A questionnaire was filled for each patient and the endoscopic findings were also recorded.

CLO tests

One biopsy was placed into the CLO-test kept at room temperature for 3 h. A positive result was recorded when pink color was seen in the gel within the time specified by the manufacturer.

Culture

Biopsies taken by endoscopy for culture were placed in normal saline solution. Each biopsy was dissected into small pieces using a sterile blade. The small pieces then were inoculated onto Chocolate agar medium with 5% defibrinated sheep blood and selective supplements (Helicobacter pylori selective supplement, SR147E, Oxoid, England). The patients were incubated under a humidified microaerophilic conditions (10% CO_2, 5% O_2, and 85% N_2) using CompyGen^{TM} Gas pack (Oxoid ltd, England) at 37^0C for 3 – 5 days and then examined for H. pylori-like colonies. The organisms were identified as H. pylori by

Gram staining, colony morphology, oxidase, catalase, and urease positive reactions.

Histology

Two antral biopsies from each patient were sent to the histopathology laboratory at Okmeydanı SSK Hospital. Sections were stained with H & E and with a modified Giemsa stain. Multiple high–power fields (HPFs) were examined by an experienced histopathologist for the detection of the presence of H. pylori and for the evolution of the inflammatory changes.

Enzyme Immunoassay

The ELISA kit SIA™ HELICOBACTER PYLORI (HM-CAP™) (Sigma Diagnostics, MO 63178, USA) was used to detect IgG antibodies to Helicobacter pylori in human serum. The test was conducted according to the manufacturer's instructions and the results were read using the Elx800 Universal Microplate Reader (Bio-Tek Instruments, Inc., USA)that measures the absorbance of the sample. The washing steps during the experiment was done using the Elx50 Auto Strip Washer (Bio-Tek Instruments, Inc., USA)

Immunoblot assay

All the ELISA positive samples were tested by the Helico-blot 2.0 system (Genelabs® Diagnostics, Singapore), which was used for detection of specific antibodies to H. pylori antigens in particular CagA (116 kDa), VacA (89 kDa) and four urease subunits (35, 30, 26.5, and 19,5 kDa). The test was performed according to the instruction manual.

The recommended criteria for determining a sample as H. pylori seropositive is any one band at CagA, VacA, or 35 kDa; or any two bands among UreA (30 kDa), 26.5 kDa, or 19.5 kDa. In general, individuals who have active

infections with H. pylori would have recently to several of these and the other bacterial proteins on the blot.

IV. RESULTS

In this series there were 33 H. pylori (+) and 12 H. pylori (-) patients according to the ELISA test. 1 (2%), 16 (36%), 5 (11%), 23 (51) patients had peptic ulcer disease, which were GU, DUE, GC, gastritis, respectively (Table 5). The prevalence of H. pylori serapositivity among these diseases was shown in table 5. PUD were not associated with seropositivity rate (χ^2 = 0.1, P= 0.01).

Whereas patients with different jobs, education level, sex, and age showed generally different prevalence level of H. pylori, no significant difference was found between these results according to the χ^2 (Chi-square) tests analyses (table 6,7,8,9). But there was a significant different prevalence level of H. pylori and socioeconomic status (P<0,05) (Table 10)

30 patients out of 45 were seropositive by immunoblot and ELISA Nine bands on the strips of these patients were commonly recognized. The band with 116 kDa was 80% was the most common band with the order of 35, 30, 26.5, 89 and 19.5 kDa (Table 11, Figure 6). No significant difference in the positive rates of these antibodies was observed between DU, GU, and G. There was an interesting result that no female individual had any antibodies to 19.5 kDa antigen. And only three patients were anti-VacA positive.

Although there was a difference in the pattern of response to the 116 kDa antigen (CagA), this was not found to be specially associated with PUD (Table 12). Statistical analysis showed no significant difference between the presence of CagA and the presence of VacA, the following factors: age, sex, education level and social economic status. These results were analyzed by means of Chi-square (χ^2).

Table 5 Correlation of H. pylori infection in 45 dyspeptic patients with histopathologic findings*

Histopathologic findings	No. of patients examined	No. of H. pylori (+) patients (%)	No. of H. pylori (-) patients (%)
Gastritis	23	16 (70)	7 (30)
Duodenal ulcer	17	13 (76)	4 (24)
Gastric ulcer	1	1 (100)	0 (00)
Gastric cancer	5	4 (80)	1 (20)

*χ^2 (Chi square): Not Significant

Table 6. Correlation of H. pylori infection in 45 dyspeptic patients with sex*

Sex	No. of patients examined	No. of H. pylori (+) patients (%)		No. of H. pylori (-) patients (%)	
Male	26	20	(77)	6	(23)
Female	19	13	(68)	6	(32)
Total	45	33	(73)	12	(27)

*χ^2 (Chi square): Not significant

Table 7. Correlation of H. pylori infection in 45 dyspeptic patients with age*

Age	No. of patients examined	No. of H. pylori (+) patients (%)		No. of H. pylori (-) patients (%)	
> 45	23	15	(65)	8	(35)
< 45	22	13	(59)	4	(41)
Total	45	33	(73)	12	(27)

*χ^2 (Chi-square): Not Significant

Table 8. Correlation of H. pylori infection in 45 dyspeptic patients with education*

Education	No. of patients examined	No. of H. pylori (+) patients (%)		No. of H. pylori (-) patients (%)	
Illiterate	11	6	(55)	5	(45)
Primary	24	19	(79)	5	(21)
Secondary	10	8	(80)	2	(20)
Total	45	33	(73)	12	(27)

*χ^2 (Chi square): Not Significant

Table 9. Correlation of H. pylori infection in 45 dyspeptic patients with occupation*

Occupation	No. of patients examined	No. of H. pylori (+) patients (%)		No. of H. pylori (-) patients (%)	
Worker	14	9	(64)	5	(36)
House wife	8	6	(75)	2	(25)
Retired	10	6	(60)	4	(40)
Others	13	12	(92)	1	(8)
Total	45	33	(73)	12	(27)

*χ^2 (Chi square): Not Significant

Table 10. Correlation of H. pylori infection in 45 dyspeptic patients with socioeconomic status*

Socioeconomic status	No. of patients examined	No. of H. p (+) patients (%)		No. of H. p (-) patients (%)	
Poor	16	9	(56)	7	(44)
Middle	29	24	(83)	5	(17)
High	0	0	(00)	0	(00)
Total	45	33	(73)	12	(27)

*χ^2 (Chi-square): $P < 0.05$

Table 11. The frequency of bands for six antibodies to different molecular weight H. pylori antigens with PUD

PARAMETERS	TOTAL	H.P.	TTWB	116 kDa	89 kDa	35 kDa	30 kDa	26.5 kDa	19.5 kDa
Number	45	33	30	24	3	17	21	19	11
Prevalence (%)	100	77	67	80	10	57	70	63	37

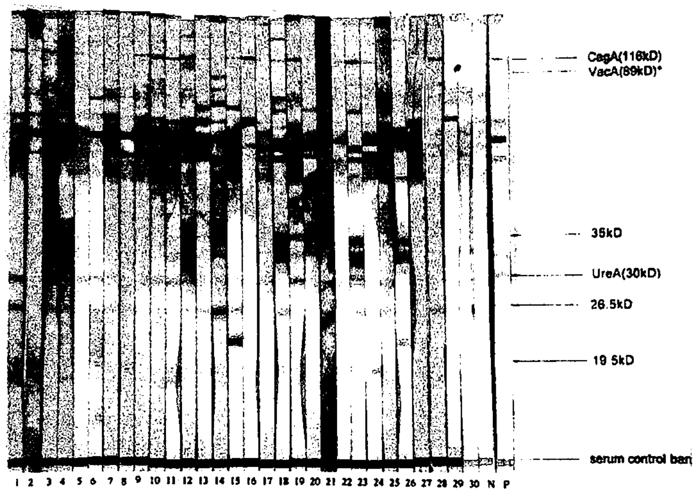
N. Patients: number of patients; H.P.: Helicobacter pylori seropositive patients; TTWB: patients assayed by western blot test

Table 12. Correlation of H. pylori anti-CagA antibodies in 30 seropositive dyspeptic patients with disease*

Disease	No. of patients examined	No. of CagA (+) patients (%)		No. of CagA (-) patients (%)	
Gastritis	14	10	(71)	4	(29)
Duodenal ulcer	13	11	(85)	2	(15)
Gastric ulcer	1	1	(100)	0	(00)
Cancer	4	4	(100)	0	(00)

*Chi-square test (χ^2). Not Significant

Figure 6 The immunoblot results obtained with sera from Helicobacter pylori seropositive patients with gastroduodenal diseases using Helico-blot 2.0 system



V. DISCUSSION

Knowledge of existence of different H. pylori phenotypes may become clinically important. One putative virulence factor of Helicobacter pylori that has been linked to the development of peptic ulcer disease is the cytotoxin-associated gene product (CagA) (56) Therefore CagA may be used as a phenotypic marker for virulence strains of Helicobacter pylori (17, 30, 40, 56). CagA has been reported to be expressed in approximately 60% of isolate of H. pylori and to be highly immunogenic (56). Whereas many studies illustrated the association between CagA and PUD (5,49,50,52,54) some Asian studies showed no association between CagA and PUD (6,29,44,56).

ELISA test and Immunoblots were used during this study. The results of present study illustrated that ELISA test is useful for the detection of anti H. pylori antibodies and immunoblot was appropriate to detect anti-CagA antibodies. The previous study demonstrates very low prevalence of VacA antigens. It may be due to low immunogenicity of VacA antigen. The prevalence of CagA in our study seems to be similar to the results in Japan. Chi-square Statistical analyses using Chi-square revealed no significant association of prevalence of anti-CagA antibodies between PUD. On the other hand it was fantastic that all 3 gastric cancer patients tested by Western blot had anti-CagA antibody. A step-by-step increase was found from gastritis to gastric cancer.

All patients were categorized according to their answers to fill up the questionnaire and we performed statistical analysis to discover whether any relationship between the parameters and the presence of anti-H. pylori & anti-CagA & anti-VacA antibodies. The result showed no relationship. Meanwhile, the number of patients below 40 ages was too low. Furthermore, when we mention that population of young individuals in Turkey is much more than that of the older ones, age seems to be highly associated with the infection.

Not any anti-19.5 kDa antibody was found among the female individual.

In this study, we describe for the first time the prevalence of H. pylori CagA or VacA antibodies in PUD from Turkey. Overall, the prevalence of CagA serum antibody was similar in patients from Turkey as compared with patients in Europe. No association between the prevalence of anti-VacA and anti-CagA antibodies was found. This is, infection with CagA positive H. pylori strains is not associated with an increased prevalence of peptic ulcer diseases. We conclude that anti-CagA antibody detection cannot be used as a marker to determine the virulence strains of H. pylori.

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