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Some Immunological and Molecular Markers of Peptic Ulcer

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To whom seek knowledge and finally to my homeland Iraq, I dedicate my work.

Abstract

Gastritis, simply, is inflammation of the gastric lining mucosa. The ulcer is an open sore or lesion on the outer (skin) or inner (mucosa membrane) surface of the body characterized by inflammatory slough off of dead tissue or rather superficial loss of tissue.

The aim of study was to understand how *Helicobacter pylori* can evade immune system through investigating the effect of cytotoxin associated gene A (CagA) on concentration of each of Interleukin 17A (IL-17A), IL-12 and IL-10. Two hundered and forty four samples were collected. The persons who take antibiotics, proton pump inhibitor (PPI) such as omeprazole, and non-steroidal anti-inflammatory drugs (NSAIDS) were excluded. Also, biopsies were taken from suspected individuals with no infection, called negative control (they were not volunteers).

Eighty two samples were selected from the mentioned number included antral biopsies and blood from patients with age mean of 32.54 year, and age range of 1 to 60 years. All samples were subjected to a primary test, rapid urease test (RUT), to confirm them whether infected or not. Molecular detection of *H. pylori* included the investigation of Phosphoglucosamine mutase (*glmM*) gene and *cagA* gene investigation has used to detect whether there were association between symptoms and *cagA* gene presence. Three cytokines (IL-17A, IL-12 and IL-10) were measured.

According to gastroduodenoscopy finding and RUT, the control, asymptomatic and symptomatic patients formed percentage of 28.05, 26.83 and 45.12 respectively. Furthermore, symptomatic patients formed the percentage of 64.86 and 35.14 for gastritis and peptic ulcer respectively. About 94.59% of symptomatic patients were *cagA* positive but about 5.41% were *cagA* negative and conversely 4.55% of

asymptomatic patients were *cagA* positive and 95.45% were *cagA* negative. Serum CagA antigen detection indicated a significant difference between asymptomatic and symptomatic groups ($p \leq 0.0001$). There were strong association between CagA antigen concentration and severity of disease. There was significant difference ($p \leq 0.001$) between peptic ulcer and gastritis. All of the interleukins were significantly higher in patients than in controls. Their levels were also significantly higher in symptomatic than asymptomatic patients. There were an inverse relationship between IL-17A, IL-12 and IL-10 levels in child and adult groups. While in adults IL-17A and IL-12 levels were higher than in child, IL-10 levels were higher in child group than adults.

As happened with CagA antigen there was deep association between interleukins levels and severity of diseases, gastritis and peptic ulcers.

The current study showed the strong association between presence of *cagA* gene and its product and peptic symptoms of Iraqi patients.

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List of Abbreviations

Ala	Alanine
ANOVA	Analysis of Variance
APC	Antigen presenting cell
avidin-HRP	Avidin horseradish peroxidase
B7-H1	B7 homolog 1
CagA	Cytotoxin-associated gene A
*cag*PAI	Cag pathogenicity island
CCR	Chemokine (C-C motif) receptor
CD	Cluster of Differentiation
CTLA-4	Cytotoxic T-Lymphocyte Associated Protein 4
DCs	Dendritic cells
ELISA	Enzyme-linked immunosorbent assay
ER	Estrogen Receptor
ERK	Extracellular signal–regulated kinases
FoxP3	Forkhead Box P3
GALT	Gut-associated lymphoid tissue
G-CSF	Granulocyte-colony stimulating factor
GEC	Gastric epithelial cell
GI	Gastrointestinal
glmM	Phosphoglucosamine mutase
Glu	Glutamic Acid
Gro-α	Growth factor alpha
hCAP	Human cationic antimicrobial peptide
HP-NAP	*H. pylori* neutrophil-activating protein
HSP	Heat Shock Proteins
hβD-2	Human β-defensin 2
IFN- γ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
Ile	Isoleucine
kb	Kilobyte
kDa	Kilo Dalton
Le^B	Lewis B antigen
LPS	Lipopolysaccharide
MALT	Mucus associated lymphoid tissue
MAP	Mitogen-Activated Protein
MHC	Major histocompatibility complex
MIP-1α	Macrophage inflammatory protein
MMPs	Matrix Metalloproteinase
MS	Multiple Sclerosis

NK	Natural killer cell
NOD	Nuclear-binding oligomerisation domain-containing proteins
NSAIDS	Nonsteroidal anti-inflammatory drugs
nTregs	Natural Regulatory T cell
OPD	O-phenylenediamine dihydrochloride
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate Buffer Solution
PCR	Polymerase chain reaction
PD-1	Programmed Cell Death Protein 1
pH	Power of hydrogen
PPI	Proton pump inhibitor
Pro	Proline
PRR	Pattern recognition receptors
RA	Rheumatoid Arthritis
RANTES	Regulated upon activation normal T Cell expressed and secreted
RUT	Rapid Urease Test
SabA	Sialic acid-binding adhesion S
SHP-2	sarcoma (Src) Homology Phosphatase 2
SP-D	Surfactant protein D
SPSS	Statistical Package for the Social Sciences
T4SS	Type four secretion system
TCR	T cell receptor
TGF-1	Transforming Growth Factor 1
Th	T helper
TLRs	Toll-like receptors
TNF-α	Tumor necrosis factor - alpha
tTregs	thymus-derived regulatory T cell
Tyr	Tyrosine
ureC	Urease Subunit Beta
UreI	Acid-Activated Urea Channel
VacA	Vacuolating cytotoxin A
WHO	World Health Organization

CHAPTER ONE

Chapter 1 Introduction

1.1.Introduction

Peptic ulcer has been an unquestionable disease of the twentieth century. It is a sore on the mucus lining of the stomach or duodenum (Amandeep *et al.*, 2012). Epidemiological data of peptic disease and its severity have been indicated the striking geographical variations in incidence and prevalence. Most common severe peptic infections are gastric ulcer, lining damage of stomach, and duodenal ulcer, almost due to excessive acid secretion of the stomach glands. Etiologically of peptic infection was often the subject of intense debate (Sunil *et al.*, 2012). What interrupt the integrity of the upper gastrointestinal tract is dependent upon the equilibrium between "hostile" factors such as gastric acid, pepsin, *H.pylori* and NSAIDS, and "protective" factors such as mucus, prostaglandins, blood flow to mucosa and bicarbonate, and the imbalance between these factors lead to damage to the stomach, which extend from gastritis to gastric cancer (Amandeep *et al.*, 2012). Several possible causes of peptic ulcers in humans, includes host genetic, bacterial and environmental causes (Susser, 1967). Some of these factors can be mentioned as sedentary lifestyle, spicy food, drugs, alcohol intake and various bacterial infections. Furthermore, several endogenous factors such as excessive peptic secretions, lipid metabolites, inflammatory mediators and reactive free radicals have been shown to induce gastric infection (Amandeep *et al.*, 2012). *H. pylori*, which its ecological niche is human and animal gastric mucosa, may be regarded as one of the most important factors in peptic infections. It could be found in the gastric mucosa of more than 50% of world's People (Go, 2002). Of the diseases caused by the bacteria under study can be mentioned gastritis, gastric atrophy, gastric ulcer, and increased risk of gastric cancer or gastric mucosa-associated lymphoid tissue lymphoma (MALT) and duodenal ulcer. One of the most important problems of infection with *H. pylori* is

that about 90% of patients infected with it shows no symptom even if they have chronic active gastritis, and this increases the risk of gastric carcinoma (Stein *et al.*, 2013). The two types of methods for investigating of *H .pylori* is noninvasive and invasive, each with its own advantages, disadvantages, and limitations. Polymerase chain reaction (PCR) technique is one of the most powerful methods for diagnosis, and allows detecting *H. pylori* in small samples with low quantity (Garza-González *et al.*, 2014). *H. pylori* avoid the innate immune system by a many different mechanisms. One of these mechanisms is an evasion of recognition by pattern recognition receptors (PRRs), proteins that recognize pathogen-associated molecular patterns (PAMP's), (PAMP's comprise a large group of microbial molecules that vary from surface parts molecules to even nucleic acids). Intuitive when PRR' s recognizes PAMP's several extracellular activation cascades will be induced by them, leading to inflammatory responses which are essential for clearance of pathogens (Lee and Kim, 2007). *H. pylori* wriggles identification by PRR's through multiple methods, such as: avoidance of recognition by Toll-like receptors (TLR's). To avoid identification by TLR's, the bacterium adjusts its surface molecules (inclusive LPS and flagellin) (Cullen *et al.*, 2011). Interestingly *H. pylori* expresses Lewis (Le) blood group antigen in the O-antigen unit of the LPS molecule which is biochemically related to carbohydrates exist in ABO blood groups and this polysaccharide area of the molecule is an obvious technique for avoiding of the innate immune response. (Appelmelk and Vandenbroucke-Grauls, 2000). This molecular mimicry, enable the bacterium evade TLR's which recognize normally detectable O-antigen as a "self" molecule (Cullen *et al.*, 2011). There are also other strategies by which *H. pylori* establishes its own persistence including the

interaction with the initiation of the adaptive immune system such as inhibiting the antigen presentation, induction of apoptosis of gastric epithelial cells and inhibiting T cell proliferation and signaling, skewing of T cell response towards thymus-derived regulatory T cell (tTreg) cells (Lina *et al.*, 2014).

Cytotoxin associated gene A toxin is the most reactive antigen recognized in the serum of some of *H. pylori* infected patients even in children under the 3 years of age (Akada *et al.*, 2014). After *H. pylori* adherence to gastric epithelial cells (GECs), CagA will translocate into cell cytoplasm, then phosphorylated and activated and interrupt various signaling pathways resulting in disabling many cellular functions and finally cell transformation (Higashi *et al.*, 2002).

Helicobacter pylori CagA also plays a key role in regulating dendritic cells (DCs) and in inhibiting CD4+ T cells differentiation towards Th1 type cells. It also plays role in inhibiting Th17 cell response (Lina *et al.*, 2014). It has shown that *cagA* gene positive strains found commonly in patients suffering of acne rosacea, stroke and coronary heart disease (Sokić-Milutinović *et al.*, 2004). In addition, it has been found that CagA resist phagocytosis and after disruption of antigen presenting cells (APCs) phosphorylated CagA surrounded by vesicles and enter the circulation in the form of exosomes and transferred to different tissues and organs to perform its harmful effects (Shimoda *et al.*, 2016).

1.2.The aim of the study

The aim of the study is to understand the ability of *H. pylori* to escape the immune system through the study of the effect of CagA on the production of each of the IL-17A, IL-12 and IL-10. Indeed, this is achieved by the following subjects:

1- Investigation the association between gastroduodenoscopy finding and presence of *H. pylori cagA* gene in Iraqi patients.
2- Measurement of serum CagA antigen levels and its association with the severity of disease.
3- Studying the influence of CagA antigen on the level of IL-17A, IL-12 and IL-10.

CHAPTER TWO

2. Literature review

2.1.Defensive aspects of gut

What exposed of the human body to the environment is not limited to the skin only, but also extends to parts of mucus layers covering respiratory, genital and gastrointestinal (GI) tracts; and these mucosal surfaces regarded as main portals for pathogens (Heike and Oliver, 2012). The lumen of the digestive tract starting from mouth to anus represents outside of the body and its mucosal surface is heavily populated with potentially pathogenic microorganisms (Adamsson *et al.*, 1998). More precisely, this tract has a surface area of about 200-300 m^2 and is colonized by 400 different species and subspecies of bacteria with $10^{13\text{-}14}$ in numbers (Savage, 1977). Therefore, the presence of a rigid defensive mechanism in site, is of obvious necessity and indeed naturally there are a set of host factors, together prevent the antigens to penetrate the epithelial barrier. These factors constitute the so-called nonimmunologic and local immunologic defense mechanisms. Nonimmunologic factors include gastric barrier, secretions, antibacterial substances (lysozymes, bile salts, and natural antibodies) indigenous intestinal flora and peristalsis, which contribute in preventing the penetration of microorganisms, enterotoxins, and other antigens across the mucosal surface and the local immunologic system, including secretory immunoglobulin A, cell-mediated immunity and other immunoglobulins. (Walker, 1976). Histologically, in general, the wall of the GI tract is composed of the four basic layers: mucosa, submucosa, muscularis, and serosa. The mucosa layer consists of layer of epithelium, lamina propria, and muscularis mucosae (Gerard and Bryan, 2012). Digestive tube beside of all of its functions can be regarded as lymphoid organ (Agarwal and Mayer, 2010) due to the numbers of lymphocytes within it, which approximately reach up to 70% of the body's immunocytes (Jung *et al.*, 2010). The lymphoid tissue of GI tract

is termed as gut-associated lymphoid tissue (GALT) which is a component of MALT (Brandtzaeg and Pabst, 2005). The cells of GALT are distributed in two forms, isolated form such as those found in both lamina propria and intraepithelial populations and in the form of aggregated lymphoid follicles such as Peyer's Patches (Lorenz and Newberry, 2004) which are concentrated in the distal ileum (Van Kruiningen *et al.*, 2002). Immune system (host defense system) is divided into innate and adaptive immune system. Innate immunity (non-specific immune system) is evolutionarily ancient and provides a rapid immune response against antigens without prior contact with the antigen but comparatively young adaptive (acquired) immune response the slower one follows the innate response, and is characterized by creation of immunological memory (Luopajärvi, 2012). The gastric immune responses involve a vast participation of innate and adaptive components (macrophages, neutrophils, epithelial, dendritic cell) and (T and B cells) respectively (Hunt *et al.*, 2015).

2.2. Gastritis and Peptic ulcer

Gastritis, simply, is the gastric lining mucosa inflaming. With regard to mucosal injury, gastritis would be classified into acute: histologically characterized by neutrophilic infiltration, and chronic: with histological hallmark of mucosal infiltrating by mononuclear cells such as lymphocytes (T and B cells), plasma cells and macrophages (White *et al.*, 2015). It may be confined to a short event (acute gastritis) or may be extended to a long duration (chronic gastritis). Peptic infection could be with abnormal appearance (symptomatic) or without (asymptomatic) (Chey and Wong, 2007). The most common symptoms are upper abdominal pain. Other symptoms may include nausea and vomiting, hypochlorhydria malaise, heartburn, fullness and flatulence (Axon, 1994). In most complex conditions it may lead to bleeding (Chey and Wong,

2007). Ulcer is an open sore or lesion of the outer (skin) or inner (mucosa membrane) surface of the body characterized by inflammatory sloughing off of dead tissue or rather superficial loss of tissue. They are mostly encountered on the skin of lower extremities and in the lining of any site of gastrointestinal tract. Among many types of mucosa membrane ulcer such as mouth, esophagus, peptic and genital ulcer, the peptic ones which include lining of stomach and/or the duodenum is often seen more severe among *H. pylori* infected patients (Chan and Graham, 2004; Bhowmik *et al.*, 2010). So peptic ulcer is an abnormal state of the gastroduodenal mucosa and defined as destruction of the gastric or duodenal mucosa and penetration of muscularis mucosae layer and finally exposure of the submucosa layer of the digestive tract (Izabel, 2011). Based on the site of ulceration, the two common types of peptic ulcer are termed gastric ulcer (in respect with stomach) and duodenal ulcer (Fashner and Gitu, 2015). Peptic ulcer is one of the world's major gastrointestinal disorders and affecting 10% of the world population (Zapata -Colindres *et al.*, 2006). Pathophysiologically, peptic ulcer disease results from imbalance between offensive and defensive factors. Acid, pepsin, NSAIDS and *H. pylori* are the offensive factors and bicarbonate, prostaglandin, nitric oxide, mucin and growth factors are regarded as defensive factors (Bansal *et al.* 2009). In the past, it had thought that ulcers were caused by spicy food and stress, but later these factors have been found to be only as exacerbating factors and the real influential factors have been identified to implicate: bacterial infection (*H. pylori*) and reaction to various medications, particularly NSAIDS. (Guariso and Gasparetto, 2012).

These two catastrophic factors beside bile-acids, pepsin, steroids, changes in gastric mucin consistency, alcohol abuse, smoking especially in people in their 60s or older (Goud, 2017), and emotional stress form

major etiological factors related with peptic ulcer disease (Kalra *et al.* 2011).

2.3.Discovery of *Helicobacter pylori*

More than 100 years ago and before the Australian pathologist John Robert Warren noted presence of curved bacillus on gastric epithelium in patients with active chronic gastritis, it was described by Walery Jaworski a Polish clinical researcher as spiral-shaped microorganisms in stomach mucosa of men, but his observation was not actually taken earnestly until the close of 1970s (Marshall and Warren, 1984; Konturek, 2003). These two Australian researchers, who manifested *H. pylori* role in gastritis and peptic ulcer disease, have been awarded "Nobel Prize in Physiology and Medicine" in 2005. The Nobel Assembly at the Karolinska Institute has honored them for their unexpected but paradigm shift discovery (Marshall and Warren, 1984; Marshall *et al.*, 1985). At that time when they declared their findings, it was believed for long time that stress and lifestyle factors were the major causes of peptic ulcer disease. Warren and Marshall (1984) disproved that tenet and it was soon clear that *H. pylori* cause more than 90% of duodenal ulcers and up to 80% of gastric ulcers. However, the clinical community, met their findings, with skepticism and a lot of criticism and because of that, it took quite a length of time for their discovery to become widely accepted. They encountered many difficulties to prove their discovery. In 1985, for instance, Marshall intentionally infected himself by swallowing the bacterial broth after undergoing gastric biopsy to be sure that he had not carried the bacterium, to show that it in fact caused acute gastric sickness (Niyaz, 2005).

In the past, it was thought that it belonged to *Campylobacter*, and was named *Campylobacter pyloridis*. Later, many studies have been conducted, and depending on structural properties and some techniques, characterize *Helicobacter* as a new genus differ from *Campylobacter*

species in some aspects, such as cellular fatty acids and major ultrastructural organization, the absence of methylated menaquinone 6 in *Helicobacter*, antibiotic susceptibilities and 5S and 16S ribosomal ribonucleic acid sequence comparison (Goodwin *et al.*,1989).

2.4.General Characterization of *Helicobacter pylori*

Helicobacter pylori is Gram-negative bacterium with a spiral appearance with S-shape includes 1 to 3 turns and rounded ends. It is about 2.5 to 3.5 μm in length, a diameter of 0.5 to 1μm and 1 to 2μm periodicity and has a smooth surface. It contains about 6 polar sheathed flagella (Pounder and Ng, 1995). The flagella have size about 3μm in length and distinctive terminal bulb (Geis *et al.,* 1993). *H. pylori* has high mobility in viscous solutions, and its flagella play an important role in this motility (Atherton and Blaser, 2009). It is microaerophilic (Bury-Moné *et al.,* 2006) and inhabit mucus layer of the gastric epithelium of many mammals and birds (Atherton and Blaser, 2009).

2.4.1.Genetic Content of *Helicobacter pylori*

Helicobacter pylori is characterized by a large diversity in strains, and many of their genes have been completely sequenced. Strain 26695, one of the more completely sequenced strains, has circular chromosome with approximately 1,660,000 base pairs, 1,590 genes and G + C content of about 39% in average (Tomb *et al.,* 1997).

2.4.2. Phosphoglucosamine Mutase (*glmM*) Gene

Phosphoglucosamine mutase *(glmM)* gene is one of the most useful genes for detection of *H. pylori* by PCR technique. In the past this gene was called *ureC* as its probable function related to urease production, but later it was revealed that *glmM* implicated in production of phosphoglucosamine mutase. This enzyme catalyzes conversion of glucosamine-6-phosphate into glucosamine-1-phosphate, which is finally converted into UDP-N-acetylglucosamine. The latter product regarded as

one of the main precursors of cell wall peptidoglycan and lipopolysaccharides (De Reuse *et al.*, 1997). *glmM* gene is considered as housekeeping gene, therefore could be used as a beneficial tool in *H. pylori* detection (Essawi *et al*., 2013)

2.5.Epidemiology of *Helicobacter pylori*

More than 50%, of the world's People which suffer from chronic gastritis are infected with this gram negative bacterium, and this represents the most recurrent chronic inflammation worldwide (Go, 2002). In 1994 The World Health Organization (WHO) regarded *H. pylori* as class I carcinogen due to its roles in gastric adenocarcinoma and MALT lymphoma besides the causing gastritis, gastric ulcers, and duodenal ulcers (Ernst and Gold, 2000). A study of its prevalence rate shows large variations from country to another and also within geographical areas (Go, 2002). The study of relationship between *H. pylori* infection and age in different countries showed that more than 80% of the people including kids and teenagers are infected with it found in developing countries than in developed countries (Yi-Chia Lee *et al.*, 2008), But in developed countries this rate decreased and approximately remains constant at less than 40% and even less in kids and teenagers than in adults (Pounder and Ng, 1995). In developing countries *H. pylori* infection rates are elevated highly during childhood and remain steady high, suggesting that *H. pylori* is acquired early during this period of life (Fiedorek *et al.*, 1991; Issa *et al.*, 2014). Whereas in developed countries (western world) the infection incidence is low during childhood and elevated by age. However this increasing rate of infections does not pass 0.5% per year. The high prevalence rates during aging reflect birth cohort impact with higher infection rates in the past (Kuipers *et al.*,1993; Parsonnet, 1995). However the prevalence rates of *H. pylori* infection in

developing countries remain comparatively stable, compared to that in the developed world which is speedily dropping (Genta, 2002). Several studies have showed that these differences in prevalence rates often affected by some factors such as socioeconomic status, age and even ethnicity race (black and Hispanic people are more infected than others). Generally, in the developing countries the prevalence is increased by crowded living statuses, poverty and low hygiene during childhood (Malaty and Graham, 1994). So it can be said that crowded families and poor hygiene may favor transmission of infection through family members. There are some possible routes of transmission of *H. pylori* such as oral-oral (perhaps by saliva or vomitus) and fecal-oral but the only proven manner is iatrogenic route which happens after endoscopy. Interindividual mode of transmission often happens either among children and adults belong to different institutions such as kindergartens, nurseries, quarantines and etc. or by clustering infection through families. Beside these manners, feces polluted water may be is a common route, particularly in geographic regions lacking water purification systems (Brown, 2000). In Iraq according to study conducted in Al-Anbar governorate in 2002, about 77% of population suffering from *H. pylori* infection and it was frequently influenced by age (Baqir *et al.*, 2002).

2.6. Colonization and Persistence of *Helicobacter pylori*

2.6.1. Urea

Avoiding gastric acidity is vital for microorganisms to colonize the gastroenteric tract (Montecucco and Rappuoli, 2001). *H. pylori* by developing multiple different mechanisms of acid adaptation are able to save itself within the bactericidal low pH environment of the lumen. Among these, the synthesis Ni_2^+-containing enzyme, urease hydrolyzes urea into ammonia and carbon dioxide. This enzyme is crucial in buffering the periplasm and probably resulting in creation of a neutral

microenvironment surround the bacteria. Despite of the 10% of urease which appears externally on the surface, due to cell lysis during culture, most of the enzyme is found inside the bacterial cytoplasm. Optimum pH of surface urease activity is between 7.5 and 8.0, and below pH 4.0, the enzyme irreversibly inactivated. Cytoplasmic urease has low activity at neutral pH, but its activity increases about 10- to 20-fold when external pH range become 6.5-5.5 and remains high down to pH 2.5 (Rektorschek *et al.,* 2000).

Urease activity is controlled by pH-gated channel UreI, in the inner membrane, which regulates the entry of the substrate, urea, to the bacterial cell in response to low pH (Pflock *et al.*, 2006). Furthermore, urease may also help to recruit neutrophils and monocytes into the inflamed mucosa and to activate production of proinflammatory cytokines (Montecucco and Rappuoli, 2001). So, urease is considered as one of the main antigens diagnosed by the human immune response to *H. pylori* (Ferrero *et al.*, 1994). Despite all these, the nature and the extent of this immune response after infection is not fully understood (Del Giudice *et al.,* 2001).

2.6.2. Flagellum and Chemiostassis

Helicobacter pylori is unable to live in an acidic environment and dies speedily in the low pH, but it is develop several mechanisms to reduce exposure to the low pH of the gastric lumen by remaining in very close vicinity to the surface of the epithelium, in which the pH is near neutral (Amieva and El–Omar, 2008). Swimming ability of most *H. pylori* bacilli confers them the capability of permanence within a narrow band of the protective mucus gel which is constantly secreted and renewed by epithelial cells of the stomach. This capability can be obtained by its polar flagella and in fact regarded as important colonization agent, since non-motile mutants are unable to colonize the

stomach. (Schreiber *et al.*, 2003). Another important beneficial factor controlling the colonization of the gastric lining by directing the bacterial movement and, of course, from the gastric lumen towards deep mucosa is the chemotactic response to the pH gradient and this assumed happened due to the difference in the permeability of the mucus to the various ions (Montecucco and Rappuoli, 2001). It has been shown that what responsible for the negative chemotaxis of *H. pylori* within pH gradients is a chemoreceptor(s). When placed in adjacent of low pH, *H. pylori* increases its swimming speed and changes its swimming pattern to boost movement away from environment with high acidity toward the most neutral one (Croxen *et al.*, 2006).

2.6.3. Adhesins

About more than 20% of *H. pylori* bacteria which are found in the stomach, adhered to mucus surface of epithelial cells (Hessey *et al.*, 1990). Electron microscopy studies of the gastric biopsy specimens showed that extensive areas of the epithelial layer have been colonized by the *H. pylori* and the latter have been extended deeply to the intracellular junctions, including cytoskeleton adjustment at the adhesion site and also some of them have been seen inside the epithelial cells. All these observations propose that adhesion mission involves interactions of specialized molecules to gastric mucosa which may lead to strong connection and modification of both the cell surface and underlying cytoskeleton. The genome sequencing of several *H. pylori* strains show that, in spite of their relatively small genomes, more than 30 genes are detected, engaged in expression of outer membrane proteins. A large number of them have been identified as possible candidates for adhesion, proposing multiple variable modes of attachment to the cell surface. The delivery of the main *H. pylori* virulence factors CagA and Vacuolating cytotoxin A (VacA) are extremely related to adhesion, suggesting an

essential role of adhesion in the delivery of toxins (Amieva and El–Omar, 2008). In addition to what was said about adhesion, the process is necessary to avoid mechanical clearance, allow holding firmly to the environment (Algood and Cover, 2006).

2.6.4. Vacuolating Cytotoxin A (VacA)

The exotoxin VacA is one of the best characterized *H. pylori* virulence factors. It was discovered in supernatants from broth cultures of *H. pylori* with its tendency to induce large intracellular vacuoles in cultured mammalian epithelial cell lines (Cover and Blanke, 2005). Toxic effects of VacA could be performed when secreted and delivered to host cell membrane in its active form. *vacA* gene expression lead to produce a 140 kDa protoxin VacA. After protein synthesis, processing events lead up to mature toxin with 88 KDa of molecular mass which is secreted into the extracellular space as a soluble protein (Ilver *et al.*, 2004). The secreted toxin can pack into water-soluble oligomeric flower-shaped complexes, and can be integrated into planar lipid bilayers, forming anion-selective membrane channels (Cover *et al.*, 1997). More than 50% of the toxin has been shown to remain associated with the bacterial surface. Interestingly, these amounts of associated VacA molecules are still functional and by direct interaction between adhered bacteria and the epithelial cell membrane are delivered to host cells (Ilver *et al.*, 2004). After internalized, VacA toxin can localize in multiple sites in the host cell, including endosomal compartments and even inner mitochondrial membrane, forming large intracellular vacuoles. VacA intoxication and anion-selective channel forming involves both epithelial cells and T-lymphocytes (Cover and Blanke, 2005). VacA by insertion into late endosomal vesicles membrane induces pores formation and chloride ions influx, causing of the ions composition alteration, and finally osmotic swelling. (Amieva and El–Omar, 2008). Actually endosomal liquid

profusion is necessary for antigen presentation, which shown to be confused by VacA. Local antigen processing inhibition could be part of a strategy, by which bacterium survive, and can significantly contribute to chronic infection of the stomach (Montecucco and Rappuoli, 2001). Also, VacA by the formation of pores in mitochondrial membranes triggers host's cell death through apoptosis. Another reported action of VacA is the ability of cause leakage of small molecules and ions, such sugars, amino acids, iron and nickel, by disabling the barrier role of tight junctions, but without serious disruptions in junction's integrity. *H. pylori* by such a mechanism could be able to acquire nutrients across an intact epithelial barrier (Amieva and El–Omar, 2008).

2.6.5. Cytotoxin Associated Antigen

Cytotoxin-associated gene A antigen, the major responsible for pathological feature of *H. pylori* infection, is a protein with molecular weight of about 120-145 kDa and its coding gene included within cag pathogenicity island (cagPAI). This bacterial onco-protein makes the *H. pylori*-mediated adenocarcinoma as the second most lethal cancer type in the world (Tohidpour, 2016). The cagPAI is the region of about 40-kb of *H. pylori* chromosome includes the mentioned gene and approximately 29 genes code for a type IV secretion system (T4SS) dependent secretion of CagA toxin (Cendron and Zanotti, 2011). CagA is the only protein known that secreted from the Cag T4SS in *H. pylori*. One of the main characteristics of CagA is its capability to interact with the host cell kinase, undergoing tyrosine phosphorylation modifications (Tohidpour, 2016). The tyrosine phosphorylation site of CagA involves conserved arrangement of amino acid motifs located in the C-terminal region of the protein and consists of Glutamic Acid (Glu) -Proline (Pro) -Isoleucine (Ile) -Tyrosine (Tyr) -Alanine (Ala) (together called EPIYA) (Fischer, 2011). Geographically and on the basis of CagA- positive strains of *H.*

pylori and amino acid sequences that flank them, four worldwide distributed characteristic types of EPIYA motifs, A,B,C and EPIYA-D, have been identified. The first two of them, –A and –B, generally are contained in all CagA-positive strains but EPIYA-C is mainly found in strains that harvested in western countries such as USA, Europe and few Asian countries such as India and Malaysia (Western type CagA), while EPIYA-D is mainly found in the Eastern countries (Far East Asian regions such as Korea, China, Japan) (Eastern type CagA) (Backert *et al.* 2010). The multiplicity of EPIYA motif numbers at the C-terminal of CagA can vary due to repetitive amino acids flanking these motifs. So this determines the EPIYA motifs variability number that carried by different CagAs (Kim *et al.*, 2006) and indeed the variance in pathogenicity of different CagA-positive strains (Basso *et al.*, 2008). For instance, transfection of human gastric epithelium *in vitro* revealed that strains with ABCCC type-CagA can trigger significant numerous genes transcription involved in gastric carcinogenesis compared to the strains with ABC-type CagA (Vaziri *et al.*, 2015). In Iraq, CagA containing *H. pylori* strains possess virulence factors comparable to those in Western countries (Hussein *et al.*, 2008). For CagA insertion into the gastric epithelial cells (GECs), *H. pylori* form a syringe-like pilus structure. It could be done by some of T4SS proteins, CagI, CagL, CagY and CagA. The latter by itself binding to integrin β1on the host cell surface, passed across the host cell membrane. Also the importance of interaction between CagA and phosphatidylserine has been shown for the injecting process (Tegtmeyer *et al.*, 2011). After delivery and tyrosine phosphorylation at motifs containing the amino acid sequence EPIYA of CagA, it can deregulate intracellular signaling pathways and thereby initiates pathogenesis (Asahi *et al.*, 2000; Umehara *et al.*, 2003). Phosphorylated CagA is able to interact with the sarcoma (Src)

Homology Phosphatase 2 (SHP-2) tyrosine phosphatase, makes it functionally active, triggering a host cell morphological change to a more motile phenotype known as the 'hummingbird phenotype' (Hatakeyama and Higashi, 2005). This cell-scattering phenotype participates in different aspects of carcinogenesis, including metastasis. CagA is also a very antigenic protein, associated with a prominent inflammatory response due to induction of IL-8 production (Lax, 2005). CagA/SHP-2 interaction also causes deregulation of cell-cycle and cellular proliferation (Zhu *et al.*, 2005). It has been found that exosomes released from CagA expressing gastric epithelial cells may reach the circulation, carrying CagA to distant tissues and organs in its active phosphorylated form. So, CagA-containing exosomes may be implicated in the expansion of extragastric disorders related with cagA-positive *H. pylori* infection (Shimoda *et al.*, 2016).

2.7. Immune Response to *Helicobacter Pylori*

2.7.1. Innate Immunity and Inflammation

2.7.1.1. Pattern Recognition Receptors:

By colonizing gastric lumen, *H. pylori* can trigger a complex of immunological response includes initiation of innate and adaptive immune systems. Infection stimulates an acute inflammatory response involving the influx of immune cells such as eosinophils, neutrophils and lymphocytes and plasma calls. This occurs as a result of the interaction of some of immune cells such as epithelial cells, DCs and macrophages with PAMPs (Lina *et al.*, 2014). Nevertheless, this acute inflammatory response seldom leads to successful eradication of pathogen (Valle *et al.*, 1996). There are several factors participate in disease outcome whose resultant is chronic gastritis, and in some cases cause the onset of more severe diseases such as peptic ulcers and gastric cancer. These factors include host factors such as genetic predisposition and immune response,

bacterial factors such as virulence factors and environmental factors such as smoking and high-salt diet (Montecucco and Rappuoli, 2001; Blaser and Atherton, 2004). Both innate and inflammatory immune responses take place soon after infection, where *H. pylori* induces the production of some cytokines such as tumor necrosis factor (TNF-α) and IL-1, IL-6 and chemokines such as IL-8, growth factor alpha (Gro-α), regulated upon activation normal T cell expressed and secreted (RANTES), macrophage inflammatory protein (MIP-1α) (Bodger and Crabtree, 1998). Inflammatory chemokines production is regulated by the transcription factor called nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), primary activation of which is believed to be mediated by epithelial cells (Baeuerle and Henkel, 1994) and bacterial proteins coded by the *cag*-PAI genes (Glocker *et al.*, 1998). *cagA* found in approximately 60-70% of strains and is known to cause more augmented inflammatory response, and therefore patients infected with a *CagA+* strain are more probable to develop severe pathologies such as peptic ulcers and gastric cancer (Covacci *et al.*, 1993; Gatti *et al.*, 2006). Some of other *H. pylori* products such as urease molecules, adhesion proteins, VacA and Heat Shock Proteins (HSP) which can effectively induce a mucosal inflammatory response, also are capable of triggering the pro-inflammatory cytokines secretion (Clyne *et al.*, 2007).

Pathogen-associated molecular patterns structures of *H. pylori* interact with PRRs such as toll-like receptor (TLRs) and nucleotide-binding oligomerization domain-containing proteins (NOD)-like receptors on innate cells such as epithelial cells, DCs and macrophages (Takeuchi and Akira, 2010). Generally the interaction between *H. pylori* and TLR conducts the pro-inflammatory cytokines production such as IL-17A, IL-12, TNF-α, interferon gama (IFN- γ), IL-1β and IL-6 (Portal-Celhay and Perez-Perez, 2006) and anti-inflammatory cytokines IL-10

and IL-4 (Ihan and Gubina, 2014). Despite of this, *H. pylori* seem to be weak agonist even with its virulent and pathogenic properties (Testerman and Morris, 2014).

The epithelial cells of stomach express TLR2, TLR4 and TLR5 (Schmausser *et al.*, 2004) which engage with PAMPs such as peptidoglycan, lipopolysaccharide (LPS), flagellin, double stranded RNA, lipoprotein, and zymosan (Mogensen, 2009; Nøorgaard *et al.*, 1995). It has been revealed that in contrast to most bacterial infections which result from implication of PAMPs with TLR4 and TLR5, *H. pylori* seem to interact with TLR2 (Mandell *et al.*, 2004) and stimulate NF-κB (Smith *et al.*, 2003). TLR2 receptors are very important in the mucosal immune response to *H. pylori* infection, because their interaction with *H. pylori* neutrophil-activating protein (HP-NAP) conduct antigen presenting cells (APCs) to produce IL-12 and IL-23 (Amedei *et al.*, 2006).

Nucleotide-binding oligomerisation domain-containing proteins play crucial role in the *H. pylori* immune response. They divided into NOD1 and NOD2 receptors (Müller and Solnick, 2011). The first group is important for innate mucosal response to *H. pylori* and is anchor for bacterial peptidoglycans such as muramyl tripeptide, the latter group has specificity to muramyl dipeptide recognition (Chamaillard *et al.*, 2003). By interaction of NOD1 with peptidoglycan muropeptides of *H. pylori*, epithelial cells induce IL-8 and NF-κB, triggering an inflammatory response (Viala *et al.*, 2004).

Another PRR is glycoprotein expressed through gastric pits at the gastric lumen called surfactant protein D (SP-D). These proteins increased during *H. pylori* infection and bind to them and decrease their motility (Moran *et al.*, 2005).

2.7.1.2.Antibacterial Peptides

Antibacterial peptides are compounds conducted by the host immune system against invader pathogens, these compounds offer their services of killing mechanism by disrupting microbial membranes, blocking of DNA or RNA synthesis and activating antimicrobial enzymes to degrade components of the pathogen. Most common host antibacterial peptides may be are defensins and cathelicidin which isolated from different sources in the body (Diamond *et al.*, 2009). Human β-defensin 2 (hβD-2) from MKN45 cell line has been shown to be induced only against *H. pylori* strains contain the *cagPAI* (Hamanaka *et al.*, 2001). NF-κB activation is fundamental for the *hβD-2* gene induction (Uehara *et al.*, 2003). Cathelicidin antimicrobial peptide found associated to *H. pylori* infection and a curative agent for chronic gastritis (Zhang *et al.*, 2016). LL-37/Human cationic antimicrobial peptide (hCAP)-18 has been reported to be the single known human cathelicidin whose production is upregulated in the gastric secretion and epithelium of *H. pylori*-infected patients (Hase *et al.*, 2003).

2.7.2. Adaptive Immune Responses

2.7.2.1.Humoral Responses

Helicobacter pylori infection stimulates strong antibody responses in the gastric mucosa in both symptomatic and asymptomatic patients, and it is known that both IgA- and IgM-secreting cells in infected subjects are higher frequencies compared to noninfected subjects (Mattsson *et al.*, 1998). However it has been shown that IgA was a major predictor of *H. pylori* seropositivity in patients with gastric cancer (Atalay *et al.*, 2002). The expression of endothelial adhesion molecules triggering trafficking of B cells to the inflamed gastric mucosa could be the reason of this difference. Further, the presence of specific serum antibodies against *H.*

pylori antigen has been utilized as a diagnostic test (Kindermann *et al.*, 2001).

2.7.2.2. Cellular Immune Responses

Cluster of Differentiation (CD**)** 4+ T-cells, the important inducer of adaptive immunity, conclude the majority of T cells (Swain *et al.*, 2012). Their surface molecule, CD4, interacts with major histocompatibility complex (MHC) II molecules normally found only on antigen-presenting cells. During TCR stimulation, naïve CD4+ T cells can differentiate into distinct lineages and secrete special sets of cytokines. In addition, there is some plasticity with respect to change in T helper cell phenotypes because of environmental stimuli (Zhou and König, 2003). Many studies have revealed their importance in the *H. pylori* infection response. They are available in the gastric antral of patients with *H. pylori* infection that show duodenal ulcers but not in the duodenum of *H. pylori* infected individuals (Lundgren *et al.*, 2005). These cells subdivided into other subsets such as T helper termed as Th1, Th2, Th17 and tTreg (Luckheeram *et al.*, 2012).

T helper 1 cells, a well described CD4+ T cell subset, are known by their ability to produce IL-2, IL-10, IFN-γ and TNF-α in response to cellular activation (Ng *et al.*, 2013; D'Elios *et al.*, 2011). The main role of them and their cytokines is the protection against intracellular bacterial and viral infections. In response to IL-12 secreted by innate immune cells after activation by pathogen, CD4+ cells would be induced to develop into Th1 cells (Hsieh *et al.*, 1993). In turn, Th1 cytokines would conduct macrophage stimulation, nitric oxide (NO) production and CD8+ T-cell proliferation, resulting in the phagocytosis and eradication of microbial pathogens (Niedbala *et al.*, 2006; Scheller *et al.*, 1997). The role of Th1 cells in disease caused by *H. pylori* was revealed that when mice with T-cells subsets deficiency had not shown gastritis progression after *H.*

pylori infection (Eaton *et al.,* 2001). This was confirmed by *H. pylori* infected mice with IFN-γ deficiency that conducts low inflammation with no gastritis development (Akhiani *et al.*, 2002). Additionally, IFN-γ+ type CD4+ T cells are the dominant subset during *H. pylori* infection (Bamford *et al.*, 1998). Some of *H. pylori* strain-virulence factors such as *cagPaI* and *vacA* which are implicated in IL-12 induction in *H.pylori*-gastritis, may be drawn immune response toward responding-Th1 (Takeshima *et al.*, 2009).

T helper 2 (Th2) lymphocytes confer immune protection against extracellular parasites such as helminthes but also can induce acute and chronic inflammatory responses versus many allergens. It has been shown that Th2 response was strongly found in patients with gastric cancer, but Th1 response was found predominantly in less severe cases, gastritis (Ren *et al.*, 2001). IL-4 is the main compound for differentiation of naïve T cells into Th2 cells (Zhu *et al.* 2005).

T helper 17 (Th17) cells are another subset belong to CD4+ T cells and encounter specific extracellular microbes (Korn *et al.* 2009) through their capacity in recruitment and activation of neutrocytes, either directly by IL-8 production (Pelletier *et al.,* 2010), or indirectly by inducing the tissue resident cells to produce IL-8 (Ouyang *et al.,* 2008). However Th17 cells had implicated as potent pathogenic factor in many inflammatory diseases and it has been suggested that Th17-mediated immune responses might be extended to several autoimmune diseases (Torchinsky *et al.,* 2009).

In terms of development and function, CD25+ CD4+ Treg cells are special T cell subset which are engaged in balancing homeostasis and immunological tolerance to self-antigen and therefore, suppressing pathological self-reactive inflammation or excessive immune responses harmful to the host (Sakaguchi *et al.,* 1995). tTregs have the power to

down-regulate the activity of most lymphoid cells as well as dendritic cells during cell-cell contact dependent mechanisms (Raghavan and Quiding-Jarbrink, 2012). Seemingly, according to their origin, there are two main types of Tregs, The thymus-derived Tregs (tTregs) also named natural Tregs (nTregs) mature in the thymus and the inducible Tregs (iTregs including Tr1 and Th3) arise from naïve T-cells in the periphery after encounter with foreign antigens (Sakaguchi *et al.*, 1995). Several studies suggest that during *H. pylori* infection, the local tTreg response protects the gastric mucosa from extreme tissue inflammation and damage, and the risk of peptic ulcer disease is inversely related to tTreg recurrences. At the same time, the obstruction of the inflammatory response carried out by tTreg leads to increase bacterial number. Moreover, the deficiency in mount a protective inflammatory response can drive to chronic infection and in some individuals to the development of atrophic gastritis and progression to gastric cancer (Raghavan and Quiding-Jarbrink, 2012).

Cluster of Differentiation 8+ T (cytotoxic) cells, like CD4+ T (helper) cells, are created in the thymus and express the T cell receptor (TCR). CD8+ T cells can bind to MHC Class I molecules presented by all nucleated cells (Zhang and Bevan, 2011). They are remarkable in immune defense versus the intracellular pathogens and in tumor surveillance (Walch *et al.*, 2014). With regard to *H. pylori* infection, samples of duodenal ulcer individuals and asymptomatic *H. pylori* carriers show a preferential production of IFN-γ by CD8+ cells than by CD4+ T cells (Quiding-Järbrink *et al.*, 2001). CD8+ cells also secrete inflammatory cytokines such as IL-17A (Caruso *et al.*, 2008). It has been shown that CD8+ cells mostly participate as memory cells in the gastric mucosa and this may have implications in progression of more severe diseases (Azem *et al.*, 2006).

2.8. Interleukin 17A

IL-17A is the member of homodimeric pro-inflammatory cytokines family (IL-17A-F), mainly secrete by activated Th17 especially type CD4+CD45RO+ memory T cells; with 35 kD molecular weight and 155 amino acids in length. IL-17A stimulates GECs and macrophages to secrete IL-8, recruiting of neutrophils via IL-8-dependent mechanism into *H. pylori*-colonized gastric mucosa to induce acute inflammatory response (Kolls and Lindén, 2004; Luzza *et al.* 2000; Mizuno *et al.*, 2005).

2.9. Interleukin-12

This heterodimeric pro-inflammatory cytokine is produced by APCs in response to intracellular microbial infection. Secreted IL-12 induces naïve T cells to differentiate into Th1 cells and IFN-γ production. The production of both cytokines not only support naïve T cells differentiation to Thl, but also inhibits Th2 responses (Airoldi *et al.*, 2002; Navaglia *et al.*, 2005). IL-12 is made of two subunits p35 (One of the IL-6 units) and p40 (One of the IL- 23 units). To accomplish a biologically active both subunits are required, forming heterodimer (p70) (Yuzhalin and Kutikhin, 2012).

2.10.Interleukin-10

Interleukin-10, the homodimer anti-inflammatory cytokine is responsible for inhibiting cell-mediated immunity and inflammation. It consists of two polypeptide chains, 160 amino acids in each one with about 17-20 kDa of molecular weight (Burdin *et al.*, 1997). It primarily produces by Th2. Nonetheless its secretion by other T helper cells, tTreg cells, CD8+ T cells, B cells, DCs, macrophages, mast cells, and natural killer (NK) cells is revealed. (Moore *et al.*, 2001). Its function is summarized in inhibition of MHC class II expression molecules on

monocytes and macrophages making these cells to limit secretion of pro-inflammatory cytokines (Couper *et al.*, 2008).

CHAPTER THREE

3. Materials and Methods

3.1.Materials

3.1.1. Equipments

The equipments used in this study with their sources are given in table (3-1).

Table (3-1): Equipments and instruments used in this study

Name of equipments	Model	Company Name	origin
Autoclave		LabTech	Korea
Cooling Eppendorf Centrifuge	Z 216 MK	Hermle labnet	Germany
Digital Sensitive Balance	TE214S	Sartorius	Germany
Horizontal Gel Electrophoresis		Cleaver scientific	UK
Power supply	EV262	Consort	Belgium
ELISA reader	16670	HumaReader HS	Armenia
Eppendorf tube (1.5ml)		Bio-Basic	Canada
Hot plate with magnetic stirrer	LMS-100	LabTech	Korea
Micro pipette 0.5-10 µl		Dragonlab	china
Micro pipette 100-1000 µl and 10-100 µl Multichannle Micro pipette		Humapette	Germany
Nanodrop	3300	Cambridge	UK
Oven	07-33406	Binder	China
PCR tube (0.3ml)		Bio-Basic	Canada
Refrigerator		Ariston	Australia
Refrigerator		Concord	Lebanon
Thermocycler (PCR device)	TC9600-G	Multigene	USA
UV Transilluminator	UVTS 100215 003	Cleaver Scientific	UK
Vortex		ROMA MB	Italy
Water bath	SW23	Julabo	Germany
Water Distillator	WD-2008f	LabTech	Korea

3.1.2. Chemicals and Biological Materials

The chemicals and biological materials used in this study and their origins are given in table (3-2).

Table (3-2): Chemicals and biological materials

Name of chemicals	Company Name	origin
Agarose	Bio-Basic	Canada
free nuclease water	Bioneer	India
Absolute ethanol	J.T.Baker	Netherland
Primers (glmM) and (cagA)	Primer	China
Tris-Borate-EDTA buffer (TBE buffer) 0.5X	Bio Basic Ink	Canada
Urea	Labochemi	India
Urea agar base	Biomark	India

3.1.3. Running Buffer (0. 5X TBE) for Agarose Electrophoresis (Bio Basic Ink / Canada)

Item	Concentration(M)	Storage
Tris base pH 6.8	0. 0445	18-25 C^o
Boric Acid	0. 0445	
EDTA	0.001	

3.1.4. Dyes

3.1.4.1. Bromophenol Blue Stock Solution (Bio-Basic/ Canada)

Item	Concentration	Storage
Bromophenol blue	0.1% (w/v)	4 C^o

3.1.4.2. Ethidium Bromide Staining Solution (Promega/ USA)

Item	Concentration	Storage

Ethidium bromide dropper bottle	0.625 µg/ml	4 C°

3.1.5.Kits

The kits used in this study with their sources are given in table (3-3).

Table (3-3): Kits used in this study with their origins

name of kit	**Description and compounds**	**Storage**	**Name of company and origin**
DNA extraction	Spin column, FATG1 buffer, FATG2 buffer, W1 buffer, wash buffer, Elution buffer, collection tube, Eppendorf tube.	Room temperature	FAVORGEN Tissue Genomic DNA Extraction Mini Kit- USA FATGK001
	Proteinase K	4 ºC	
PCR Master mix	1X Master Mix Composition dNTPs, MgCl2, buffer components and stabilizers as well as two commonly used tracking dyes for DNA gels. On a 1% agarose gel in 1X TBE, Xylene Cyanol FF migrates at ~4 kb and Tartrazine migrates at ~10 bp	-20 ºC	Biolabs- England 0231612

IL-17, IL-12 and IL-10 sandwich ELISA kit	Detection antibody, Capture antibody, Human Interleukin standard, Avidin Peroxidase conjugate, OPD, Bovin Serum Albomenm. Phasphate Buffer Solution, Citrate Phosphate Buffer	-20 ºC	Pepro tech - UK IL-10: 900-M21 IL-12: 900-M96 IL-17: 900-M84
Anti cagA sandwich ELISA kit	antibody precoated plate/ 8×12 Human CagA Standards/ 2 vial Biotinylated antibody(1:100)/ 1vial Enzyme conjugate(1:100)/ 1vial Enzyme diluent/1vial Antibody diluent/1vial Standard diluent/1vial Sample diluent/1vial Washing buffer (1:25)/ 1vial Color Reagent A/1vial Color Reagent B/1vial Color Reagent C/1vial	4/-20℃	MyBioSource/ USA MBS262126
DNA Ladder	13 bands (suitable for sizing linear and double-stranded DNA fragments ranging from 50 bp to 1000 bp.).	4℃	Intron / South Korea 10416014

3.1.6.Primers

Primers used in this study were synthesized by Realgen (Korea). The name, sequence and product size are given in table (3-4).

Table (3-4): The gene, name, sequence and product size of primers used in this study.

Gene	Name of primer	Sequence of primer 5'-3'	Product Size (bp)	Reference
glmM	*glmM* -F	AGCTTTTAGGGGTGTTAGGGGTTT	294	(Abu-Almaali *et al.*, 2012)
	glmM -R	AAGCTTACTTTCTAACACTAACGC		
cagA	*cagA*-F	GATAACAGCCAAGCTTTTGAGG	349	(Mansour *et al.*, 2010)
	cagA-R	CTGCAAAAGATTGTTTGGCAGA		

3.2.Methods

3.2.1. Collection of Samples

The samples were collected in Kerbala (Al Hussein Educational hospital and Al-Kafeel hospital) and in Babylon (Merjan hospital) provinces, during the period from December-2016 to March-2017. Informed consent was obtained from all patients. The gastroduodenoscopy was described by the gastroenterologist. All samples were collected and analyzed under aseptic conditions. A questionnaire was prepared to obtain the host information of the patients as shown in the appendix (Questionnaire form). Samples were included gastric biopsies and about 8-9 ml of blood, then serum was separated by centrifugation. Two biopsies from antrum had taken; one of them was used firstly in RUT for primary detection of *H. pylori*, and then was transformed to saline containing tube beside another piece. Blood

samples were used to immunoglobulin G test and for comparison of serum levels of some interleukins, which assumed to be different in intact persons, asymptomatic and symptomatic patient. Studied interleukins included IL-17A and IL-12 as proinflammatory cytokines and IL-10 as an immune suppressing cytokine (Wong *et al.*, 2001). Patients with malignancies, gastric surgery, Cardiovascular and autoimmune disease (Jukic *et al.*, 2017) and who take antibiotic, PPI such as omeprazole, and NSAIDS such as aspirin, diclofenac, ibuprofen *etc.* have been excluded (Pandya *et al.*, 2017). In the current study patients with abnormal gastroduodenoscopic finding but negative RUT results also have been excluded.

In this study, two types of measurements were taken into consideration: comparing between healthy persons (controls) and infected individuals, and also, among patients themselves, those with normal gastroduodenoscopic finding and those with abnormal gastroduodenoscopic finding. Due to the presence of two types of *H. pylori*, *cagA* positive and *cagA* negative, we collected biopsies of two types of patients as probably associated with the role of this gene to the severity of peptic infections; biopsies from patients with normal finding of gastroduodenoscopy called positive asymptomatic and from those with abnormal finding of gastroduodenoscopy (in this study gastritis (inflammation, friability,edema, punctuate hemorrhage and nodularity, peptic ulcers) called positive symptomatic. Additionally, there were biopsies taken from suspected persons with similar symptoms (reflux, abdominal pain and dyspepsia), but have not gastric infection, called negative control (Eskandari-Nasab *et al.*, 2013). So, there are two types of control: negative for either person who is infected with *H. pylori* or not and asymptomatic for the probably absence or presence of *cagA* gene in positive patients.

3.2.2. Preparation of Urea Agar media

Urea agar base, was prepared based on Factory instructions and then 40% urea was filtered with micro filter paper, added to medium and mixed well, then poured into Eppindorf tube (1.5ml) before being solidified (Midolo and Marshall, 2000).

3.2.3. Detection of *Helicobacter pylori* by Rapid Urease Test (RUT) medium

Rapid Urease Test was performed in the endoscopy room immediately by placing the antral biopsy into Eppendorf tube contains solidified 1.5 ml of urea agar base media and waited about 30 min. However, color changing takes time from a few minutes to 24 hours. Color changes from yellow to pink indicated positive result and unchanged color regarded as a negative result (Yakoob *et al*., 2006).

3.2.4. Extraction of DNA from Biopsy Specimens

Each frozen biopsy specimen was thawed, crushed; genomic DNA was then extracted directly using favorgen tissue genomic DNA extraction mini kit, as described by the manufacturer.

3.2.4.1. DNA extraction protocol

1- The tissue was cut up into 25 mg, and placed in 1.5 ml Eppindorf tube and grinded into small pieces, then 200 µl of FATG1 Buffer was added and mixed well by same micropestle.

2- Twenty µl Proteinase K was added, mixed by vortex, and incubated at 60°C until completely lysed.

3- Samples were vortexed for 15 sec. then 200µl FATG2 buffer was added. Samples were mixed thoroughly by vortex and incubated at 70°C for 10min.

4- Then, 200µl absolute ethanol was added and mixed again thoroughly.

5- The mixture was pipetted into a 2 ml collection tube. Then mixture was maximum speed centrifuged for 1 min.; flowed liquid then discarded.

6- Five hundred µl W1 buffer was added, centrifuged for 1 min. And the flowed liquid discarded.

7- Seven hundred and fifty µl of wash buffer was added. Then the mixture was centrifuged for 4 min. by maximum speed, and the flowed liquid discarded.

8- The spin column was transferred into a new 1.5 ml eppendorf tube (Elution Tube), and 200 µl elution buffer was added for elution.

9- The tubes were left for a short period of time for at least 2 minutes to ensure that the elution buffer is completely absorbed. Then centrifuged for 2 min at 14000 rpm; the purified DNA eluted in the bottom of the tube.

3.2.5. Determining of DNA Concentration and Purity

The concentration and purity of DNA samples were determined by using Nano drop Instrument (A micro volume measurement platform).

3.2.6. Protocol of Gel Electrophoresis

3.2.6.1. Agarose Gel Electrophoresis (Sambrook *et al.*, 1989)

The well-forming comb was placed in position on casting tray. The open ends of the casting tray were sealed with removable rubber. Melted agarose concentration 0.8% (for genomic DNA) and 2% (for PCR product), provided with 0.5 µg/ml of ethidium bromide dye, and was poured into the casting tray until the agar surrounded the teeth. The gel was hard for about 20 minutes. When the gel was solid, the comb was removed carefully and then rubbers were removed.

The buffer chamber was filled with the 0.5 X TBE buffer. The buffer was added to cover the gel. The agar was placed in the buffer chamber.

The wells were filled with 7 µl of samples with 3 µl of loading buffer to be tested and 50-1000 bp DNA ladder as a size marker in parallel with the sample. The lid was closed and voltage (70 V) applied for 20 min (for genomic DNA) and 1.5 hours (for PCR product).

The power was turned off when the bromophenol blue dye was 5 cm apart and the gel was removed.

3.2.6.2. Agarose Gel Photodocumentation

Agarose gel was placed above the UV transilluminator device, and then the gel was exposed to UV light 254 nm. Then, a picture was taken.

3.2.7. Polymerase Chain Reaction Amplification

Table (3-5): Master Mix composition of Monoplex PCR Working Solution for *glmM* gene in each PCR tube

Component	Conc. (µl)	Final conc. (µl)
Master Mix	12.5	1x
DNA	5	variable
Primer	4	0.4 µM
Deionized water	3.5	---
Total volume	25	---

Table (3-6): Master Mix composition of Monoplex PCR Working Solution for *cagA* gene in each PCR tube

Component	Conc. (µl)	Final conc. (µl)
Master Mix	12.5	1x
DNA	3	variable
Primer	2	0.4 µM
Deionized water	7.5	---
Total volume	25	---

3.2.8. Thermal Cyclic Conditions

The PCR technique conditions of Abu-Almaali *et al*. (2012) and Mansour *et al* (2010) first used to amplification of *glmM* and *cagA* genes

respectively, but to obtain more preferable results optimization has been done.

3.2.8.1.Detection of *Helicobacter pylori* by Housekeeping Gene

To detect the presence of *H. pylori*, the housekeeping *glmM* gene was investigated (Essawi *et al.*, 2013). Monoplex PCR protocol for *glmM* gene detection showed in table (3-7)

Table (3-7): Cycling conditions of *glmM* gene amplification

Cycle	Step	Temperature (°C)	Time
1	Initial denaturation	95	5 min
38	Denaturation	92	30 Sec
	Annealing	53	40 Sec
	Extension	72	40 Sec
1	Final extension	72	5 min
-----	Hold	4	-

3.2.8.2. Investigation of the *cagA* gene

Monoplex PCR Protocol was used to distinguish *cagA* positive *H. pylori* from *cagA* negative *H. pylori*. Cycling conditions of the gene amplification showed in table 3-8.

Table (3-8): Cycling conditions of *cagA* gene amplification

Cycle	Step	Temperature (°C)	Time
1	Initial denaturation	94	5 min
35	Denaturation	94	1 min
	Annealing	53	1 min
	Extension	72	1 min
1	Final extension	72	5 min
-----	Hold	4	-

3.2.9. Enzyme-linked Immunosorbent Assay (ELISA) Protocols

3.2.9.1. Anti-human Cytotoxin Associated Gene A (CagA) ELISA

1. One hundred µl of Serum samples and CagA standard samples was added to each well, and was incubated, at 37℃ for 90 min.

2. After 2 times washing of plate, 100µl of biotinylated human CagA antibody liquid was added to each well, plate was sealed and incubated in incubator at 37℃ for 60 min. The plate was washed 3 times.

3. One hundred µl of enzyme-conjugate liquid was added to each well, and plate was sealed and incubated in incubator at 37℃ for 30 min

4. After 5 times washing of plate, 100µl of enzyme-conjugate liquid was added to each well, plate was sealed and incubated in dark incubator at 37℃ for 30 till color development.

5. One hundred µl of color reagent liquid was added to individual well and incubated in incubator at 37℃. The chromogenic reaction controlled within 30 min until became darker.

6. One hundred µl color reagent C (reaction stopping reagent) to each well (including blank one) was added and mixed well, and the results were recorded by absorbance reading at 450 nm within 10 min.

3.2.9.2. Enzyme-Linked Immunosorbent Assay Protocol for Interleukins

For detection of IL-17A, 12 and 10 in serum samples, commercially available Peprotech Human IL-17A, 12 and 10 Mini Elisa Development Kit was used.

3.2.9.2.1. Plate Preparation

1. In first day 100 µl of capture antibody at 0.5µg/ml in phosphate buffer solution (PBS) was added to each ELISA plate well and incubated overnight at room temperature.

2. In next day the plate was washed 4 times using 300µl/well of wash buffer before incubation with 300 µl/well of Block Buffer for 2 hours at room temperature. Then the plate was washed 4 times.

3.2.9.2.2. Enzyme-Linked Immunosorbent Assay Steps

1. One hundred µl of standard and serum samples added to each well for 2 hours at room temperature followed by 4 washes.

2. Detection was conducted by adding 100µl of detection antibody conjugated with biotin at a concentration of 0.25µg/ml in diluent for 2 hours at room temperature. The plate was then washed 4 times

3. One hundred µl of diluted avidin-HRP in diluent was added to wells and was incubated for 30min at room temperature.

4. After final 4 times of washing, 100µl of OPD Liquid Substrate was added to wells and was incubated at room temperature for color development.

5. Fifty µl of 14% H_2SO_4 as stop solution was added to each well and the results were recorded by absorbance reading at 495nm.

3.2.10. Statistical Analysis

Statistical analysis was done by using the software statistical package for social sciences (SPSS; version 18). The results were given as mean ± standard deviation (Mean ± S.D). Statistical analysis for the significance of differences of the quantitative data was done by using ANOVA test for single factor means. Unpaired, Unequal Variances, Student‘s t test used for determination of significant differences between means of different interleukins used in this study and CagA concentrations in serum. The probability levels were indicated as follows (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; and ****$p < 0.0001$, and similarly for other symbols) (Newman *et al.,* 1997).

CHAPTER FOUR

4. Results and Discussion

4.1. Demographic Distribution According to Age and Gender

Eighty two samples were obtained out of the 245 samples. Patients included in the current study were with age mean of 32.54 years, 39 (47.56 %) of them were males and 43 (52.44 %) were females. Their ages ranged from 1 to 60. Figure (4-1) shows the demographic distribution of male and female patients according to age groups.

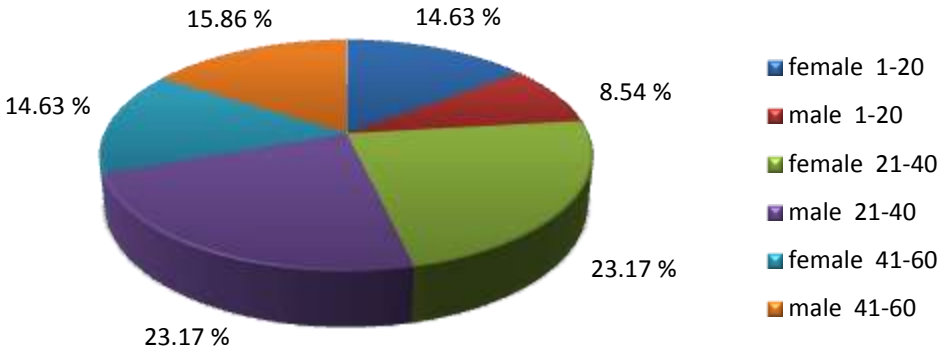

Figure 4-1: the demographic distribution of male and female patients according to age groups.

4.2. *Helicobacter pylori* Detection

4.2.1. Rapid Urease Test Detection

All samples were subjected to a primary test, RUT, to confirm them whether infected or not (Figure 4-2). Actually RUT false positive is uncommon. Patients with excessive salivation or with alkaline bile reflux from the duodenum into the stomach may conduct false positive. Contamination of small gastric biopsies during gastric endoscopy may cause the elevation of pH greater than 6 on the surface of biopsies. In this situation, weak positive reaction in RUT may occur, particularly if reagent do not include acidic buffer (pH <6.0). In addition, proton-pump inhibitors may cause decreasing of gastric acidity (achlorhydria) which

causes the subsequent superficial colonization of the gastric mucosa with other urease producing microbes (e.g., *Proteus mirabilis*, *Klebsiella* or *Candida*). Generally, these organisms give positive results with RUT about 24 hours after biopsy insertion into the urea agar base media. Additionally, when the gastric mucosa infected with other *Helicobacter* species, false-positive results may occur. On the other hand, the incidence of achlorhydria gives rise to false-negative urease test results in both biopsy and breath tests because luminal pH of 7.0 can conduct extremely high pH microenvironment close to the bacteria, this cause *H. pylori* destroying by its own urease activity (suicide). This case is revealed by the urea breath test; about 30% of patients taking normal-dose PPIs reversion to a negative breath test, although low numbers of bacteria are still present in the gastric mucosa (Midolo and Marshall, 2000).

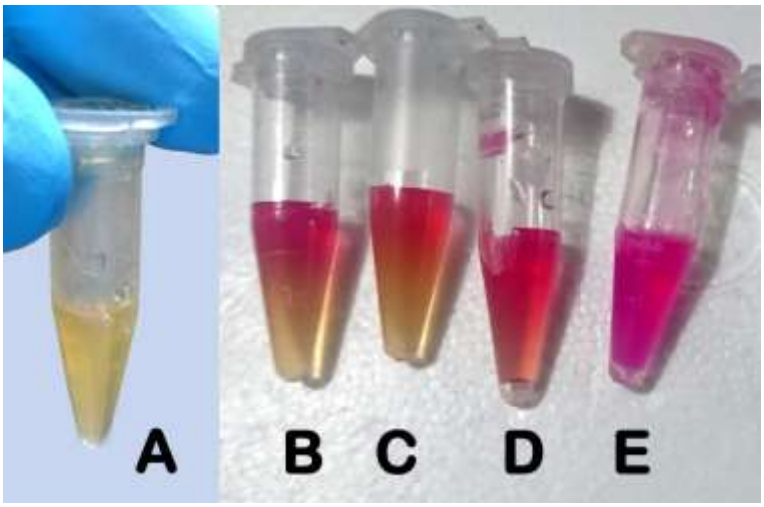

Figure 4-2: Rapid urease test results. A: negative B and C: after 10-20 min. D: after 1-2 hours. E: after 10 hours.

Table 4-1 shows the clinical characteristics of control and patients, according to gastroduodenoscopy.

Table (4-1): Total clinical characteristics of control and patients according to gastroduodenoscopy.

Status	**No.**	**Percentage (%)**
Control (negative)	23	28.05
Asymptomatic (positive)	22	26.83
Symptomatic (positive)	37	45.12
Symptomatic finding	**No.**	**Percentage (%)**
Gastritis (G)	24	64.86
Peptic ulcer (PU)	13	35.14

4.2.2. Molecular (PCR) Detection

4.2.2.1. Concentration and Purity of DNA

By using Nano-drop device, the concentration and purity of extracted DNA from biopsies were measured as shown in table 4-2. The purity ranged from 1.9 to 2.1.

Table (4-2): Concentration and Purity of Extracted DNA

Sample no.	**Concentration µg/ml**	**Purity (A260/A280)**
1	87.95	1.98
2	58.20	1.96
3	146.70	1.93
4	51.35	1.99
5	215.5	2.10
6	74.05	1.90
7	50.30	1.93
8	108.35	1.96
9	102.75	1.95
10	116.20	1.96

4.2.2.2. Phosphoglucosamine mutase gene (*glmM*) detection

Selected samples were used for second and final detection step of *H. pylori* presence. It is accomplished by the PCR technique using *glmM* gene with a product of 294 bps. Figure (4-3) shows the PCR results. All

samples of asymptomatic and symptomatic patients were contained the gene.

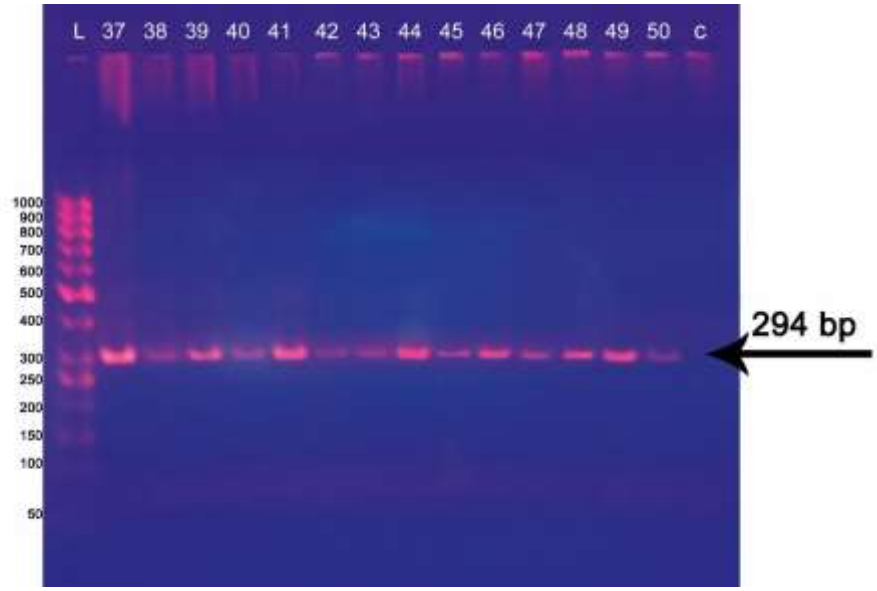

Figure 4-3: Agarose gel electrophoresis for PCR product of *glmM* gene (*ureC*). 2% agarose, visualized under UV after staining with ethidium bromide at 6volt /cm for 1 hours. Lane L is DNA ladder (bp). Lanes 37 to 50 are patients. Lane C is negative control.

The *glmM* gene is highly conserved and could be used to *H. pylori* identification in gastric biopsies. Although it has been shown that *ureA* has a sensitivity and specificity of more than 90% (Sugimoto *et al.*, 2009), the *glmM* gene has superior sensitivity than the *ureA* gene (Brooks *et al.*, 2004). One of the features of utilization the *glmM* gene in *H. pylori* identification in the gastric biopsies is its absolute sensitivity and specificity, since it presents detection rate of 10 to 100 *H. pylori* cells which is even better than histopathology (Essawi *et al.*, 2013).

4.2.2.3. Cytotoxin Associated Gene A (*cagA*) Detection

The third step of the study was to detect whether samples contain *cagA* gene or not. This step was also applied on all three groups of samples (control, asymptomatic and symptomatic). Figure (4-4) shows a

product size of 349 bp as the PCR results. The results of current study were in lined to those of Mansour *et al.*, (2010).

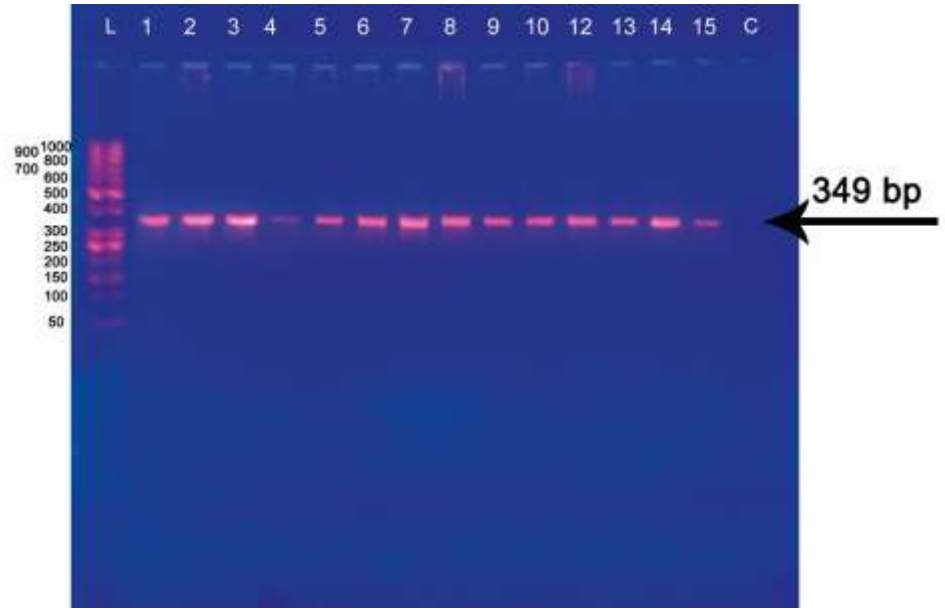

Figure 4-4: Agarose gel electrophoresis for PCR product of *cagA* gene. 2% agarose, visualized under UV after staining with ethidium bromide at 6volt /cm for 1 hours. Lane L is DNA ladder (bp). Lanes 1 to 15 are patients. Lane C is negative control.

4.2.2.4. Distribution of *cagA* Gene Among Patients Groups

The results of PCR technique show a strong association between the presence of *cagA* gene and gastroduodenoscopy finding (table 4-3). In contrast to Mansour *et al.*, (2010) results, the current study results were related to symptoms. Approximately all patients showed various infection symptoms, but some of them were intact.

Table (4-3): Distribution of *cagA* gene in patients groups.

		Positive		Negative	
Groups	**No.**	**No.**	**Percentage%**	**No.**	**Percentage%**
control	23	0	0	23	100
asymptomatic	22	1	4.55	21	95.45
symptomatic	37	35	94.59	2	5.41

4.3.Sero-Immunological Test

4.3.1. Detection of CagA protein in serum of patients by ELISA

In the current study, results of serum CagA antigen detection indicated a significant difference between asymptomatic and symptomatic groups. In symptomatic patients the mean concentration was 540.11 ± 167.34 pg/ml while in asymptomatic patients it was 266.18 ± 65.81 pg/ml (Figure 4-5).

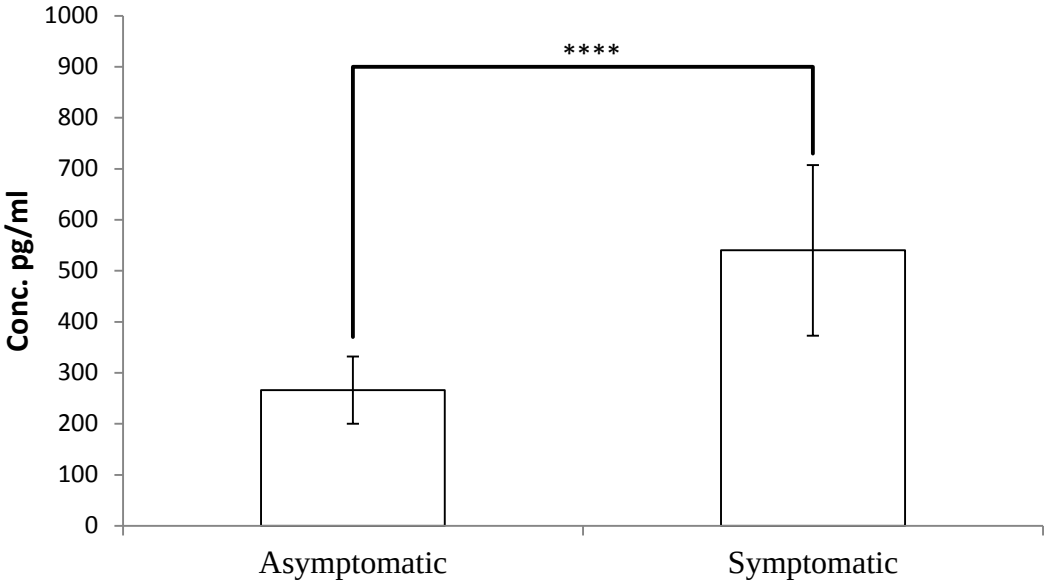

Figure 4-5: Concentrations of CagA protein in the asymptomatic and symptomatic groups (The significance value was indicated as * between asymptomatic and symptomatic patients. The levels of probability were indicated as follows ****p < 0.0001).

By comparing a presence of the gene and its product with gastroendoscopic finding, it was found that there was relationship between CagA antigen and severity of disease. Serum toxin levels were significantly higher in peptic ulcers (645.31 ± 158.43 pg/ml) than gastritis (483.13 ± 99.79 pg/ml) (Figure 4-6).

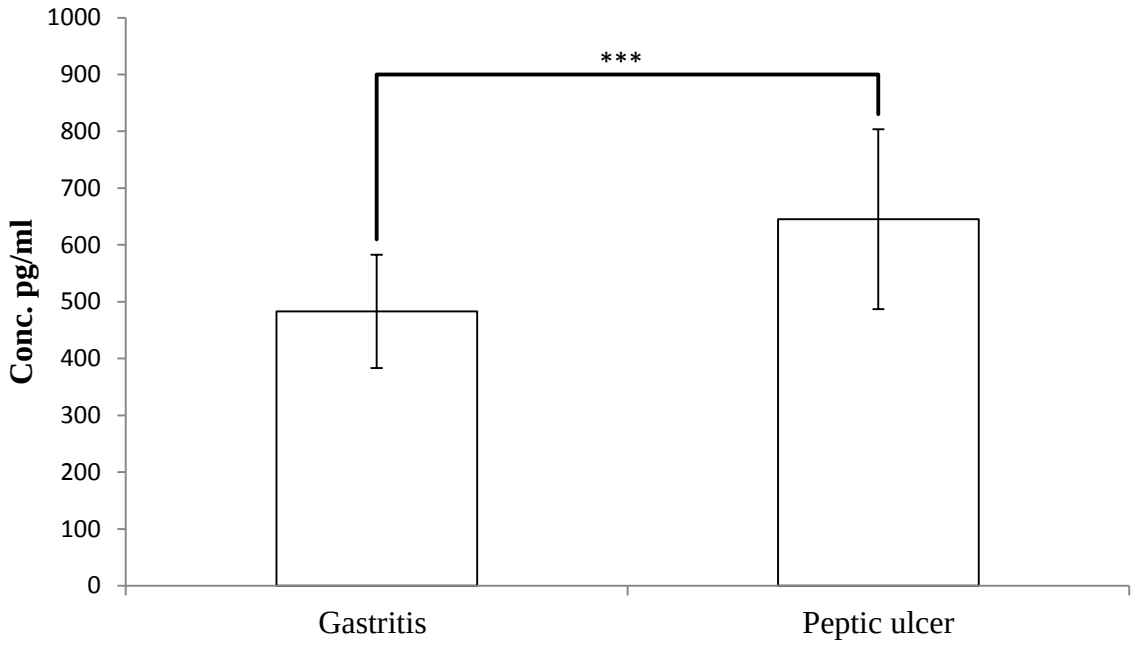

Figure 4-6: Concentrations of protein in two types of disease appeared in symptomatic patients of this study (The significance value was indicated as * between gastritis and peptic ulcer patients. The level of probability was indicated as ***P ≤ 0.001).

4.3.2. Serum cytokine levels measurement

4.3.2.1. Interleukin 17A

To investigate the role of IL-17A in the patients with peptic ulcer, the serum levels of this cytokine have been measured. The mean level of IL-17A was about (318.25 ± 106.00 pg/ml) in patients group and (38.17 ± 13.87 pg/ml) in the control group. The level of IL-17A in the patients group was significantly higher than in control group (Figure 4-7).

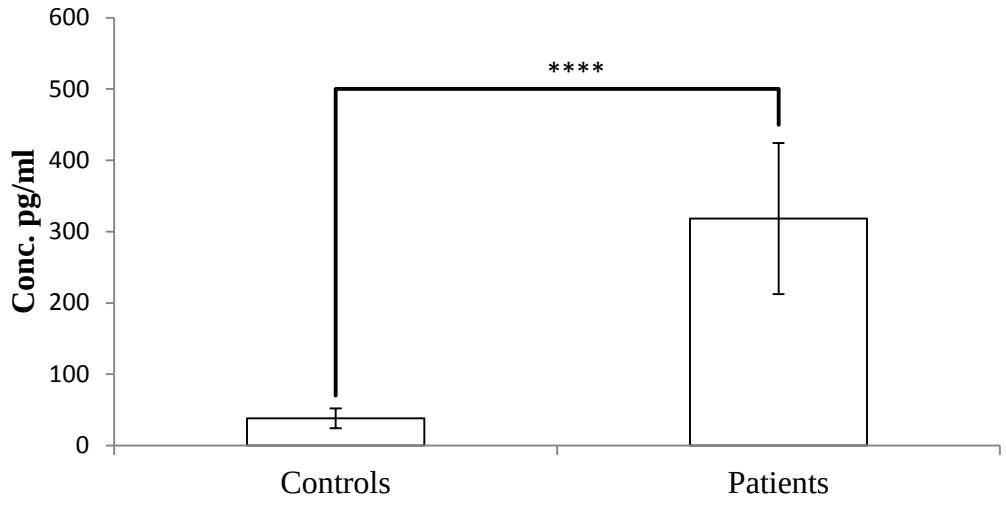

Figure 4-7: Concentration of IL-17A in control and patient groups (The significance value was indicated as * between controls and patients. The level of probability was indicated as **** P ≤ 0.0001).

In this study, serum level of IL-17A in asymptomatic and symptomatic patients also has been investigated. In patients with abnormal gastric finding (symptomatic) means level was (350.41 ± 114.91 pg/ml) and in patients with normal gastric finding (asymptomatic) was (264.18± 84.87 pg/ml). thus the level of IL-17A in symptomatic group was significantly higher than those of asymptomatic group (Figure 4-8).

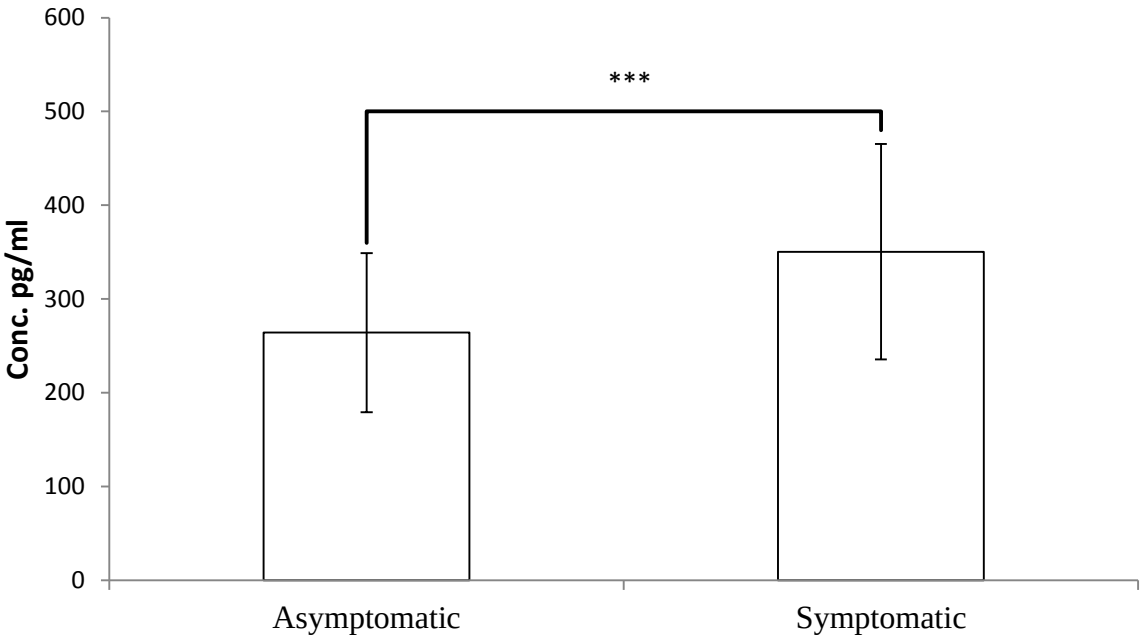

Figure 4-8: Concentrations of IL-17A in the asymptomatic and symptomatic groups (The significance value was indicated as * between asymptomatic and symptomatic groups. The level of probability was indicated as ***P ≤ 0.001).

Interleukin 17 levels in different disease types of current study were rated. By comparing the mean concentration of IL-17A between gastritis infected patients and peptic ulcer infected groups, the elevated level was observed in peptic ulcer patients (433.38 ± 97.86 pg/ml) than in gastritis patients (305.46 ± 71.05 pg/ml) (Figure 4-9).

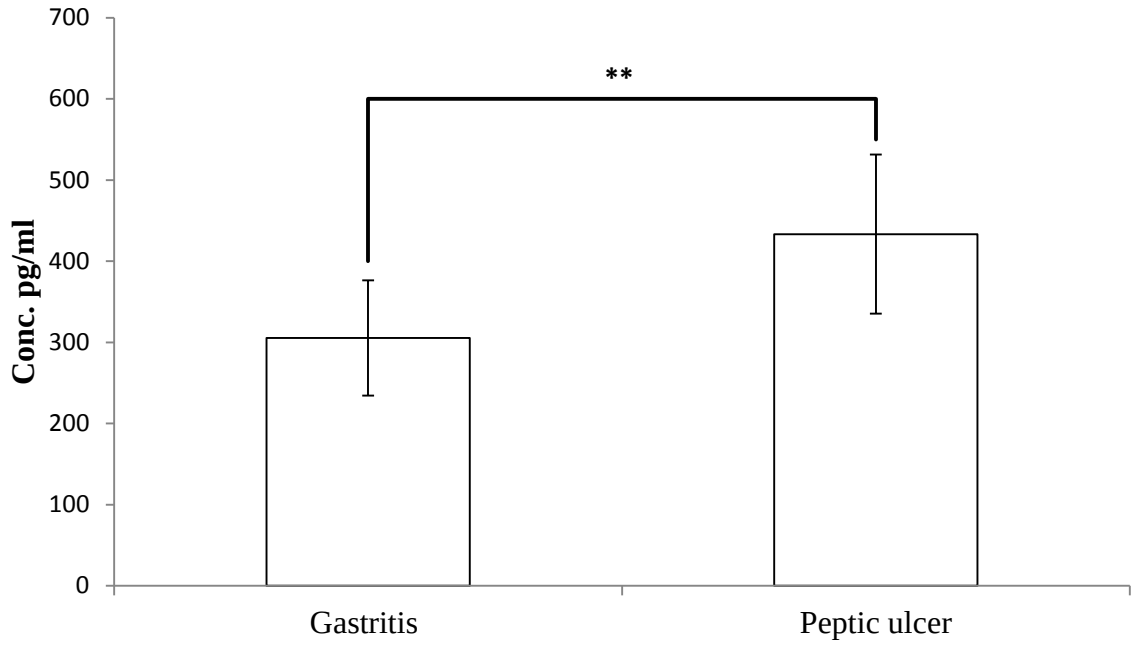

Figure 4-9: concentrations of IL-17A in gastritis and peptic ulcer disease of symptomatic patients (The significance value was indicated as * between gastritis and peptic ulcers. The level of probability was indicated as ** $P \leq 0.01$).

In control samples the serum levels of IL-17A were approximately equal in females and males. In asymptomatic and symptomatic groups the levels were significantly lower in males than in females. They were 206.63± 59.45 pg/ml and 288.83 ± 39.08 pg/ml in asymptomatic group and were 320.05 ± 93.07 pg/ml and 390.25 ± 100.34 pg/ml with significant differences between males and females of symptomatic group. Klein and Flanagan (2016) also found that the levels of serum IL-17A were higher in females than males (Figure 4-10).

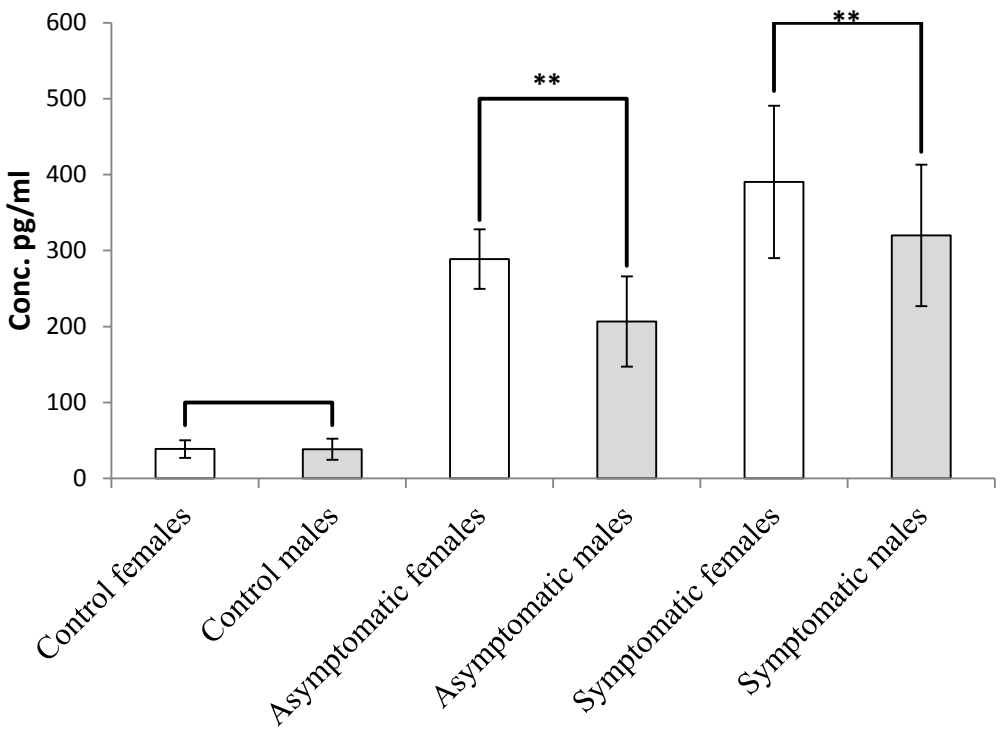

Figure 4-10: Concentrations of IL-17A in males and females of three groups under study. (The significance value was indicated as * between males and females of controls, asymptomatic and symptomatic patients. The level of probability was indicated as **P ≤ 0.01).

Males and females differ in their immunological responses to foreign and self-antigens and show distinctions in innate and adaptive immune responses. Certain immunological sex differences are present throughout life, whereas others are only apparent after puberty and before reproductive senescence, suggesting that both genes and hormones are involved (Klein and Flanagan, 2016).

With respect to the hormonal factor estrogen has been shown to modulate all subsets of T cell that include CD4+ (Th1, Th2, Th17, and tTregs) and CD8+ cells. Estrogen modulates IFNγ-secreting Th1 cells by enhancing IFNγ expression in both human and mice, which are

potentially mediated by direct interaction of estrogen receptor (ER) with estrogen-response element in the promoter region of the *Ifnγ* gene and/or up-regulating Th-1-specific transcription factor T-bet. ERα-deficient mice have decreased IFN-γ+-secreting cells in lymph nodes, suggesting estrogen-driven Th1 cell responsiveness is dependent on ERα-mediated signaling. High levels of estrogen (e.g., Level in pregnancies) are known to skew the immune response from Th1 (IFN-γ) to Th2 (IL-4). The effects of estrogen on Th17 subset have also been reported, albeit with varied response to estrogen depending on the experimental conditions. In periodontal ligament cells culture, addition of estrogen enhances IL-1β-mediated IL-17F production. In adult cystic fibrosis male mice, estrogen increases the severity of pneumonia, in part by increased Th17-regulated inflammation. However, it has also been shown that estrogen deficiency in postmenopausal women is associated with increased IL-17A levels. Estrogen also promotes the expansion and frequency of tTreg cells, which play a critical role in downregulating immune responses and upregulating the expression of forkhead box P3 (FoxP3), programmed cell death protein 1 (PD-1), and cytotoxic T-lymphocyte associated protein 4 (CTLA-4) via ERα-mediated signaling. Protective effects of estrogen in autoimmune conditions such as Multiple sclerosis (MS) and Rheumatoid arthritis (RA) are believed to be due to a combined result of estrogen-mediated tTreg expansion and activation (Khan and Ansar, 2016). It has been revealed that estrogens by modulating B lymphocytes increase IgG and IgM production in both males and females, directly and through a potentiating effect of IL-10 from monocytes (Oertelt, 2012).

Serum levels of IL-17A compared in the three age groups of asymptomatic and symptomatic patients, and there were found interesting results. In asymptomatic patients there were no significant differences

found between age groups. In symptomatic patients the interleukin levels were significantly lower (293.18 ± 63.34 pg/ml) in the first group of age than in the second group of age (400.59 ± 111.66 pg/ml). Although the concentrations were higher in 41-60 (325.56 ± 75.00 pg/ml) than1-20, there were no significant differences between these groups (figure 4-11).

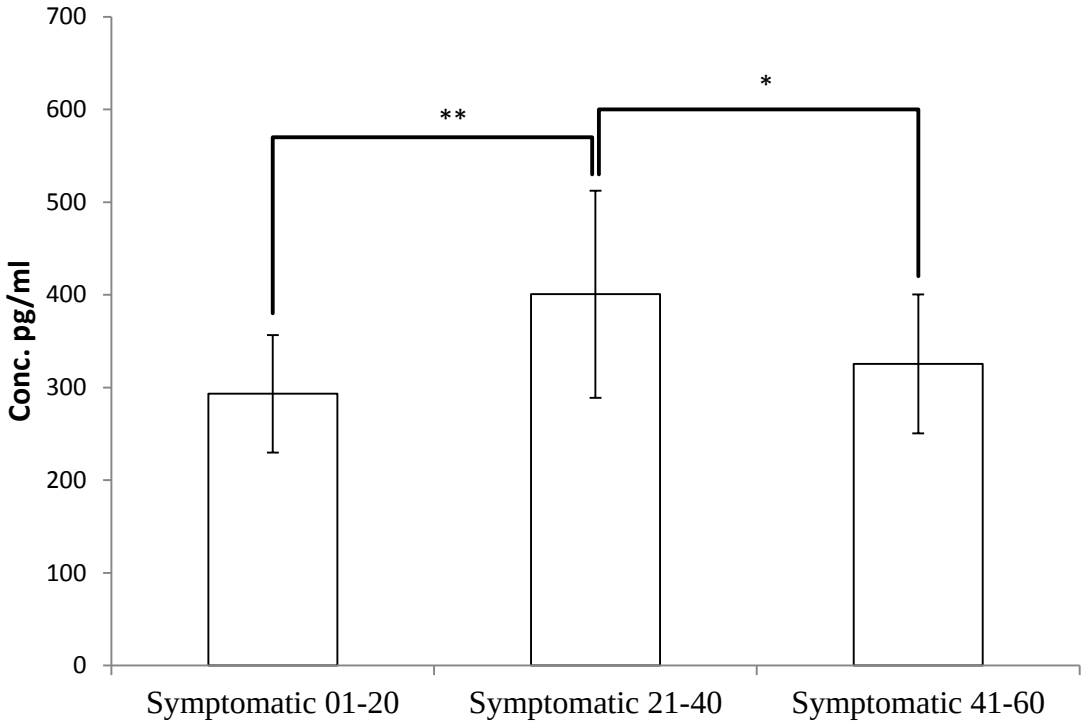

Figure 4-11: Concentrations of IL-17A in the three age groups of symptomatic patients (The significance value was indicated as * between tree age groups. The level of probability was indicated as *P ≤ 0.05 and **P ≤ 0.01).

4.3.2.2.Interleukin-12

The results of IL-12 were widely similar to the results of IL-17A. Interleukin-12 levels in patient with peptic diseases caused by *H. pylori* also compared to healthy persons. The mean value of IL-12 in patients was (385.29 ± 146.95 pg/ml) but in control (17.04 ± 7.32 pg/ml) with high significance (Figure 4-12).

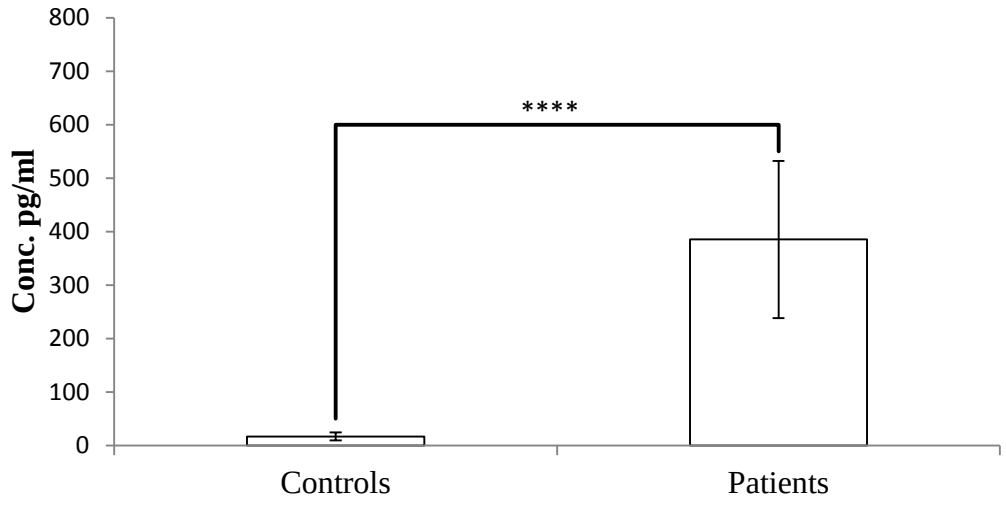

Figure 4-12: Concentrations of IL-12 in control and patient groups (The significance value was indicated as * between controls and patients. The level of probability was indicated as ****P ≤ 0.0001).

A study of the relationship between asymptomatic and symptomatic groups showed differences in the levels of underwent interleukin in serum of patients. In patients with symptomatic peptic disease mean level was 423.32± 162.21 pg/ml and in patients with normal gastric finding (asymptomatic) was 321.32 ± 113.67 pg/ml. Thus the levels of IL-12 in symptomatic group were significantly higher than in those of the asymptomatic group (Figure 4-13).

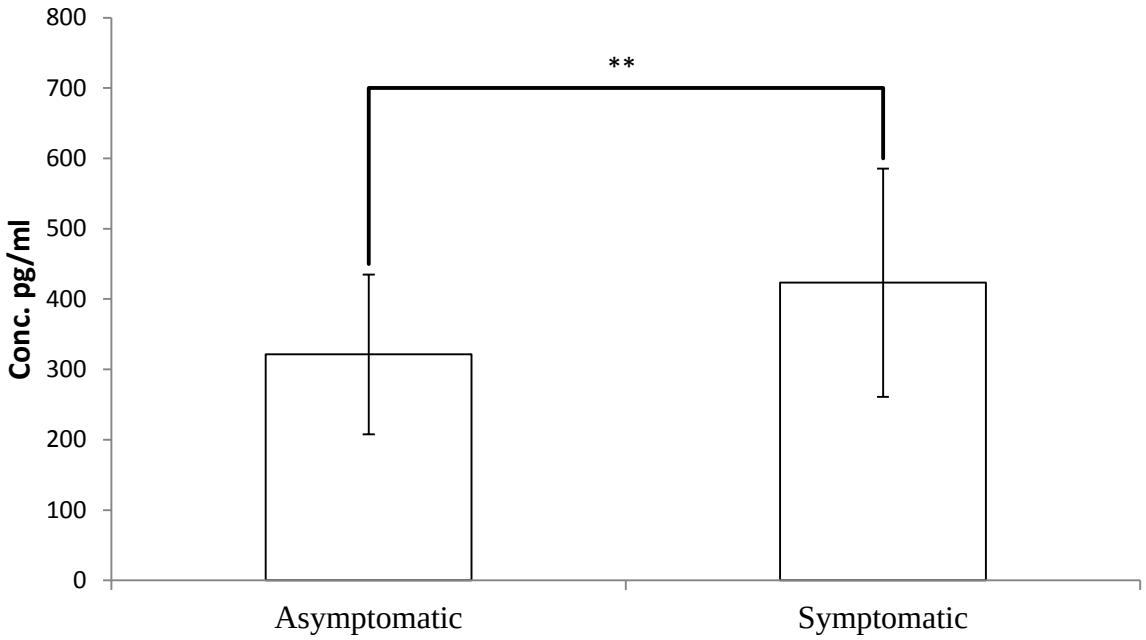

Figure 4-13: Concentrations of IL-12 in the asymptomatic and symptomatic groups (The significance value was indicated as * between asymptomatic and symptomatic groups. The level of probability was indicated as **P ≤ 0.01).

Additionally, interleukin-12 levels were checked in serum of patients with different *H. pylori* infections involved in this study. They were significantly different from each other, where in peptic ulcer the mean value was 538.00 ± 181.58 pg/ml but was 378.80 ± 116.46 pg/ml in patients with gastritis (Figure 4-14).

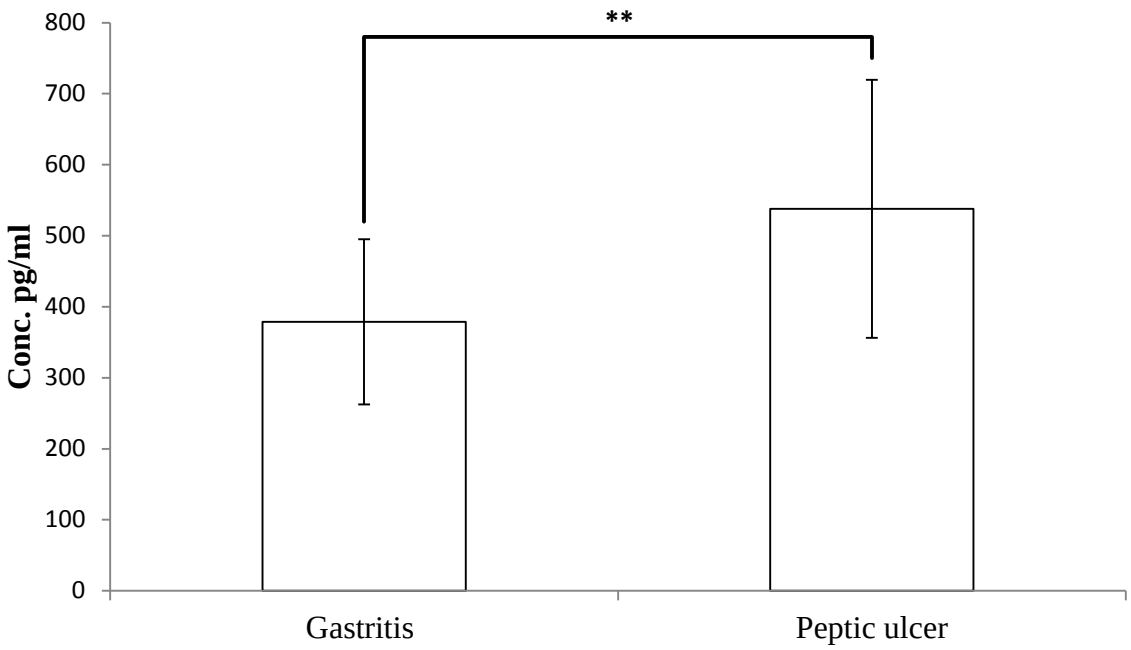

Figure 4-14: concentrations of IL-12 in gastritis and peptic ulcer of symptomatic patients (The significance value was indicated as * between gastritis and peptic ulcers. The level of probability was indicated as **P ≤ 0.01).

Data analysis of control showed different IL-12 levels in serum of females and males. IL-12 Levels were higher in females (18.77 ± 7.91 pg/ml) than in males (15.25 ± 3.77 pg/ml), but the difference did not reach significant level ($p \geq 0.50$). In asymptomatic group IL-12 levels were significantly higher in females (355.75 ± 74.07 pg/ml) than in males (254.50 ± 87.05 pg/ml). Also in symptomatic group IL-12 levels were significantly higher in females (477.44 ± 168.46 pg/ml) than in males (382.10 ± 120.58 pg/ml) (Figure 4-15). There were no significant differences among comparisons between the sex for each age group of asymptomatic patients.

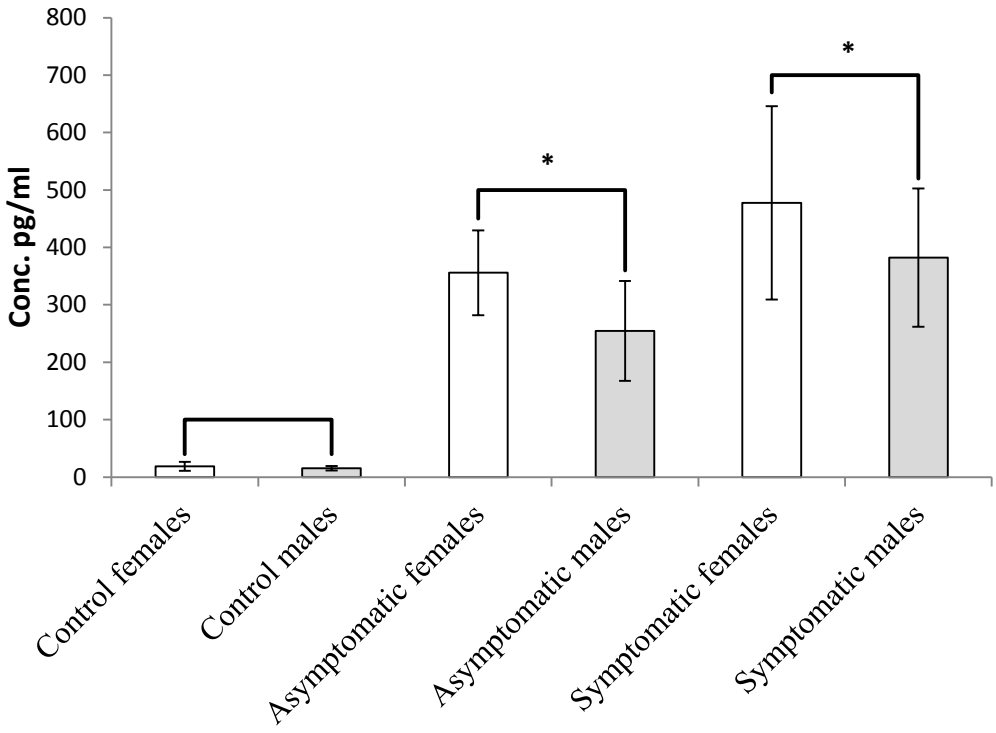

Figure 4-15: Concentrations of IL-12 in males and females of three groups under study. (The significance value was indicated as * between controls asymptomatic and symptomatic patients. The level of probability was indicated as *P ≤ 0.05).

In line with the results of IL-17A, the mean concentrations of IL-12 were lower in 1-20 age groups than in 21-40 age groups of symptomatic cases.

The concentration mean of symptomatic patients was significantly lower in 1-20 group (356.91 ± 73.87 pg/ml) than in 21-40 group (493.59 ± 185.40 pg/ml). There were no significant differences between two age groups 21-40 and 41-60, (371.78 ± 69.04 pg/ml). There were no significant differences between 1-20 and 41-60 age groups (Figure 4-16).

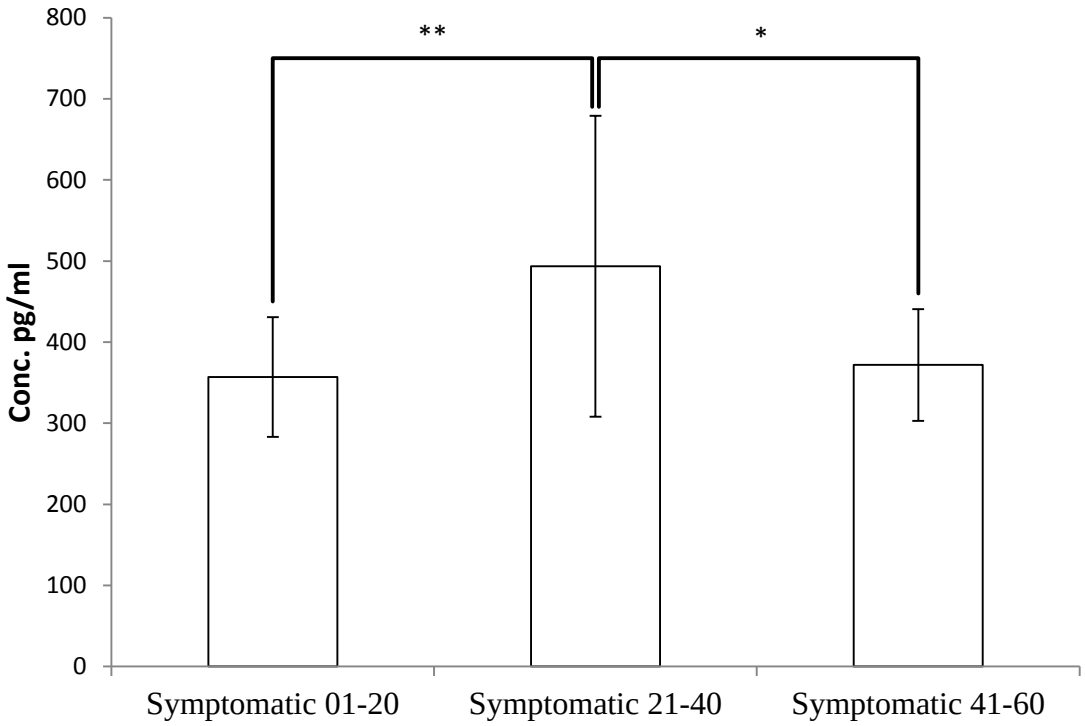

Figure 4-16: Concentrations of IL-12 in the three age groups of symptomatic patients (The significance value was indicated as * between tree age groups. The level of probability was indicated as *P ≤ 0.05 and **P ≤ 0.01).

4.3.2.3.Interleukin-10

In this study, the role of IL-10 has comprehended by its concentration measurement in serum of individuals suffering from *H. pylori* infection and comparing with that in controls serum. The mean concentrations of IL-10 were 853.34 ± 369.18 pg/ml in infected group, compared to control (23.22 ± 8.50 pg/ml) (Figure 4-17).

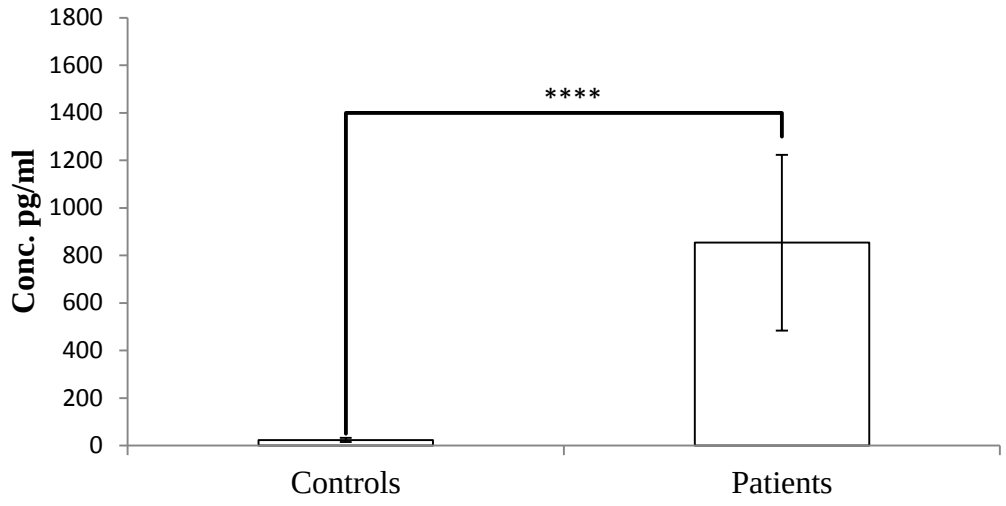

Figure 4-17: Concentrations of IL-10 in control and patient groups (The significance value was indicated as * between controls and patients. The level of probability was indicated as ****P ≤ 0.0001).

As probable relationship between type of infection and incidence of *cagA* gene in symptomatic samples, IL-10 serum levels have also been investigated in symptomatic and asymptomatic patients. The mean levels were 604.18 ± 170.27 pg/ml in asymptomatic individuals, and 1001.49 ± 355.06 pg/ml in symptomatic individuals. The concentration levels were significantly higher in symptomatic than those of the asymptomatic group (Figure 4-18).

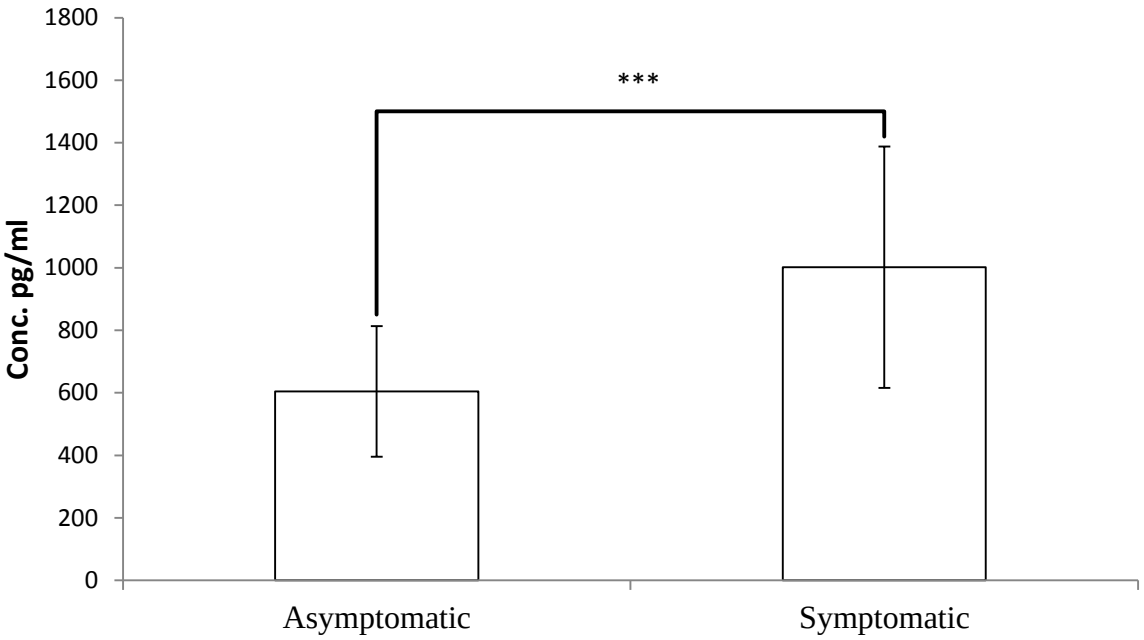

Figure 4-18: Concentrations of IL-10 in the asymptomatic and symptomatic groups (The significance value was indicated as * between asymptomatic and symptomatic groups. The level of probability was indicated as ***P ≤ 0.001).

The results of IL-10 concentration levels in probable *H. pylori* related diseases of this study (gastritis, peptic ulcer), were similar to IL-17A and IL-12 and even the serum levels of CagA protein. The mean concentrations were significantly higher in serum of peptic ulcer (1217.38 ± 349.69 pg/ml) compared to gastritis (881.2 ± 298.86 pg/ml) (Figure 4-19).

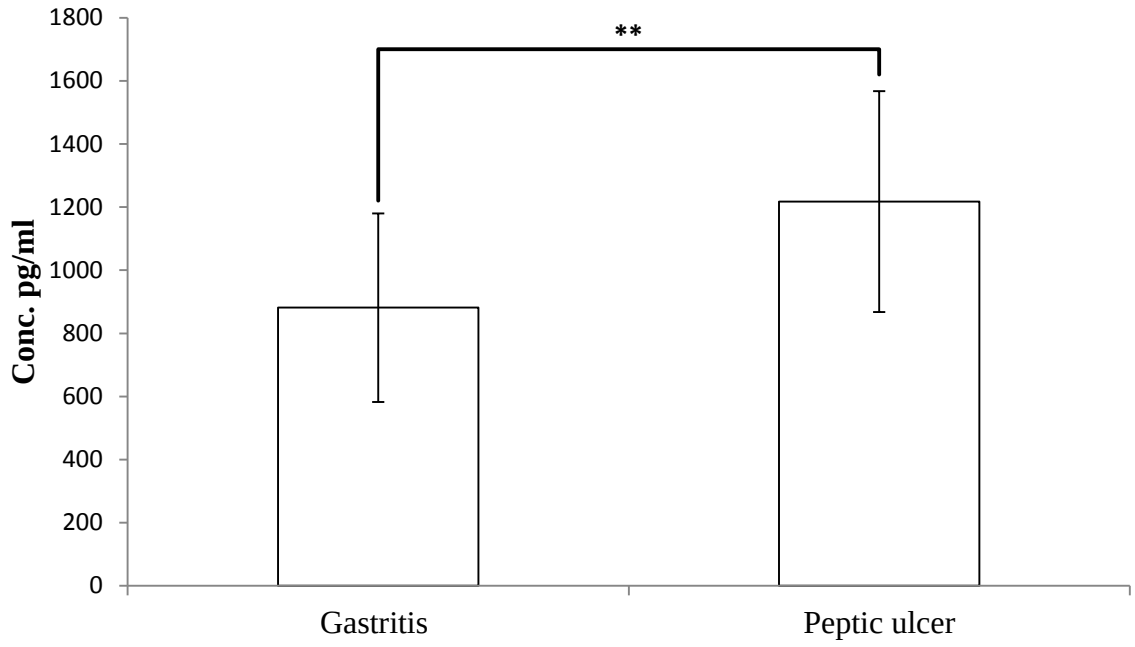

Figure 4-19: concentrations of IL-10 in two types of disease of symptomatic patients (The significance value was indicated as * between gastritis and peptic ulcers. The level of probability was indicated as **P ≤ 0.01).

Furthermore, there were differences in serum levels of IL-10 in females and males. Generally, in intact persons the mean concentrations were higher in females (24.93± 8.67 pg/ml) than in males (20.56 ± 2.83 pg/ml), but without significant difference. However the significant difference has become clear in females and males of asymptomatic and symptomatic cases. In asymptomatic females interleukin levels were 659.50 ± 151.35 pg/ml but were 498.56 ± 131.07 pg/ml in males; and in symptomatic females were 1201.56 ± 321.82 pg/ml and 849.05± 304.67 pg/ml in males (Figure 4-20).

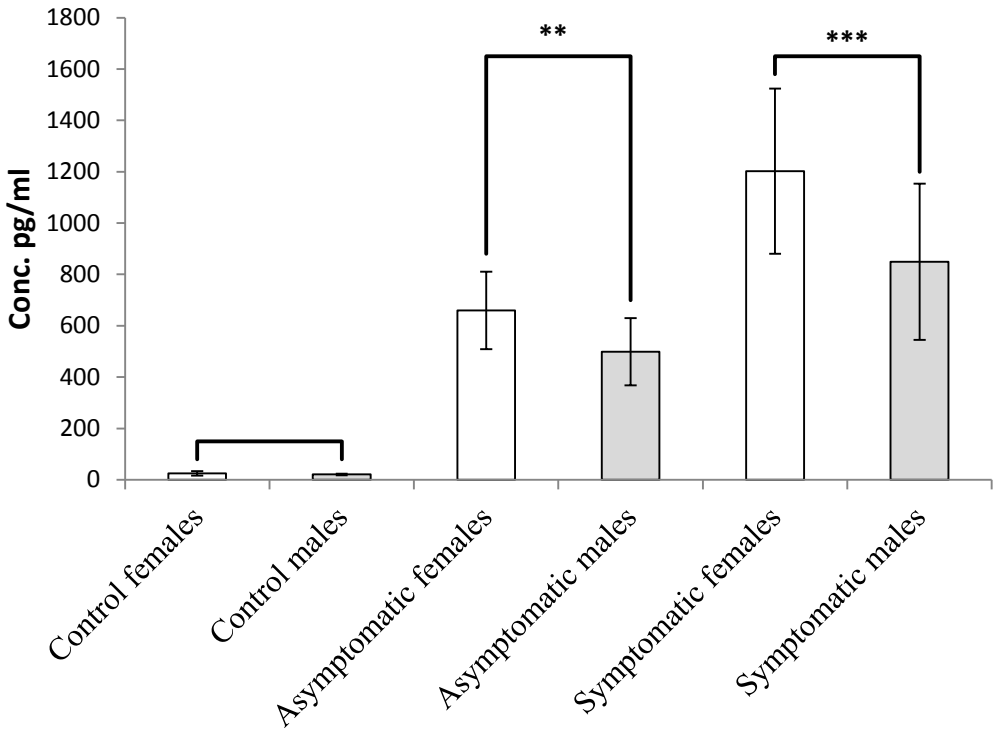

Figure 4-20: Concentrations of IL-10 in males and females of three groups under study. (The significance value was indicated as * between males and females of controls, asymptomatic and symptomatic patients. The level of probability was indicated as **P ≤ 0.01 and as ***P ≤ 0.001).

In contrast to both IL-17A and IL-12 the mean concentrations of serum IL-10 were significantly higher in the first age group than in the second and third age groups. While in asymptomatic age groups (as in both IL-17A and IL-12) there were no significant differences between levels, in symptomatic age groups there were high significant differences between age groups. In symptomatic patients, the interleukin levels were 1266.64 ± 204.86 pg/ml in 1-20 group, 973.41 ± 393.06 pg/ml in 21-40 age and 730.44 ± 157.67 pg/ml in 41-60 group (Figure 4-21).

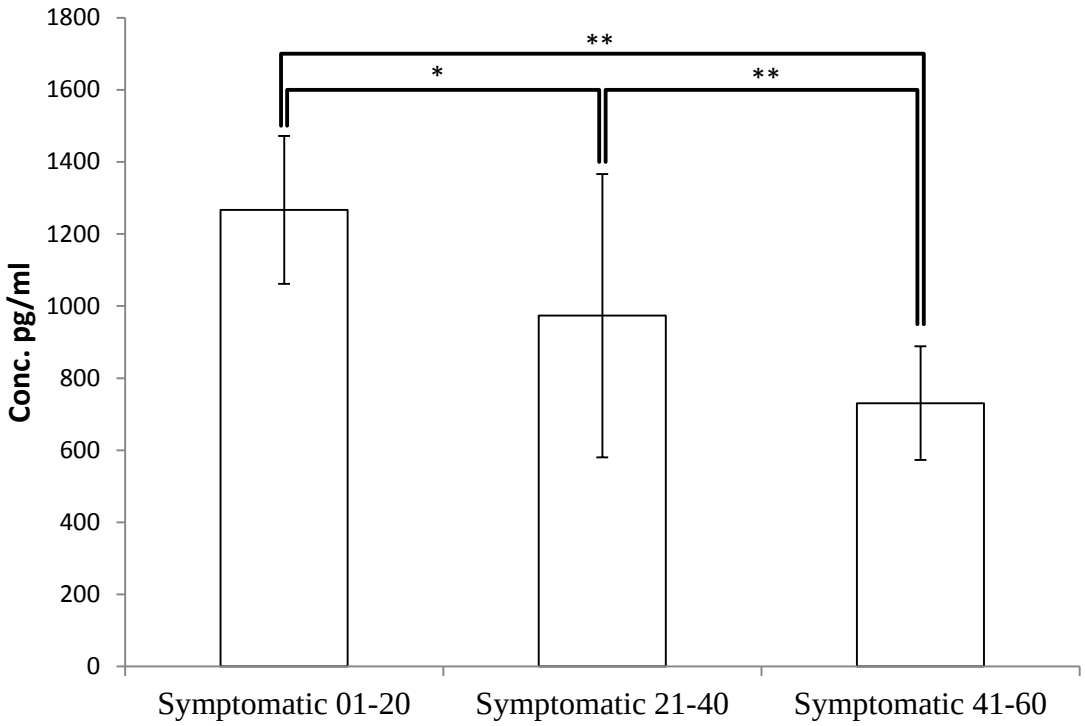

Figure 4-21: Concentrations of IL-10 in the three age groups of symptomatic patients (The significance value was indicated as * between each two age groups. The level of probability was indicated as *P ≤ 0.05 and **P ≤ 0.01).

4.3.2.4. Correlation coefficient

In the current study there were high correlations between all three interleukins and CagA protein levels in serum of both gastritis and peptic ulcer. Following tables show these correlations (table 4-4, 4-5, 4-6 and 4-7).

Table (4-4): Correlation between CagA antigen and IL-17A, IL-12 and IL-10, in patients with gastritis.

Type of disease	Toxin	Cytokine	No.	correlation	p-value
Gastritis	CagA	IL-17A	24	0.52	0.03*
		IL-12	24	0.46	0.04*
		IL-10	24	0.54	0.02*

*** P-values ≤ 0.05 is a highly significant**

Table (4-5): Correlation between CagA antigen and IL-17A, IL-12 and IL-10, in patients with peptic ulcer.

Disease severity	Toxin	Cytokine	No.	correlation	p-value
Peptic ulcer	CagA	IL-17A	13	0.77	0.02*
		IL-12	13	0.74	0.03*
		IL-10	13	0.84	0.01**

*** P-values ≤ 0.05 is significant**
**** P-values ≤ 0.01 is a highly significant**

Table (4-6): Correlation between each two interleukins in patients with gastritis.

Disease severity	Cytokine 1	Cytokine 2	No.	correlation	p-value
Gastritis	IL-10	IL-12	24	0.25	0.22
	IL-10	IL-17A	24	0.20	0.33
	IL-12	IL-17A	24	0.94	0.001***

***** P-values ≤ 0.001 is a highly significant**

Table (4-7): Correlation between each two interleukins in patients with peptic ulcer.

Disease severity	Cytokine 1	Cytokine 2	No.	correlation	p-value
Peptic ulcer	IL-10	IL-12	13	0.72	0.04*
	IL-10	IL-17A	13	0.72	0.04*
	IL-12	IL-17A	13	0.90	0.01**

*** P values ≤ 0.05 is a significant**
**** P-values ≤ 0.01 is a highly significant**

With regard to aim of this study, it needed to find how the genetic predisposition and the immune response, bacterial factors and environmental factors play role in peptic disease. Many studies nominate microbial factors and especially *H. pylori* as most effective factor for these diseases (Mustafa *et al.,* 2015). Because infection will be acquired during babyhood, and if would not be treated, the host could carry the pathogen even for life, employing an innate and adaptive immune response which is unable to enucleate the pathogen (Cellini, 2014).

To find the relationship between *cagA* gene and symptoms of disease, its product was measured in serum of symptomatic and asymptomatic patients. In contrast to approximately all studies that had detected anti-CagA antibody in serum of patients as the evidence for the CagA toxin presence, in the current study CagA antibody rather than CagA antigen is used in sandwich ELISA technique for detection of CagA protein in serum of patients. So this step can be regarded as the novel method, at least in our county. Franceschi *et al.* (2009) showed that there is mimicry between CagA antigen and antigens found in the atherosclerotic plaque occurred in tibial arteries. So, in *H. pylori* infected patients, intensive anti-CagA response to the CagA antigen may also include the protein localized in coronary atherosclerotic plaques and cause coronary instability. On the other hand anti-CagA immunoglobulin secreted against the CagA is of type G (IgG) which may cause false positive results when it needs to be investigated, because anti-CagA IgG remains for long periods may be extended for some years after *H. pylori* eradication (Shiota *et al.*, 2010). Additionally, false negative results may happen in new infection while the anti-CagA IgG levels are not sufficiently high to be investigated in serum (Miftahussurur and Yamaoka, 2016). For these reasons, in this study, anti-CagA (as its high specificity to CagA) has been used for detection of CagA protein in serum.

Navaglia *et al.* (1998) found significant association between peptic ulcer diseases and incidence of *cagA* containing *H. pylori;* and patients with GagA have a significantly increased level of anti-CagA antibody. Another study published by Rudi *et al.* (2000), pointed out that most patients with peptic ulcer diseases, infected by *cagA* positive strains, but on the contrary *cagA* negative strains don't induce significant signs of peptic infections. On the other hand, there are some studies that contradict

these findings. For instance, in Turkey, two reports provided similar results claiming that high serum level of anti-CagA is not related to severity of disease, and other bacterial factors may play a role in severity of *H. pylori* related pathology. They found elevated serum levels of anti-CagA antibody in both asymptomatic and symptomatic patients (Serin *et al.,* 2003; Abasiyanik *et al.,* 2002). In Chile the research conducted by Figueroa *et al.* (2002) presented results were mimicking to the result in current study (figure 4-5), but in their study the asymptomatic individuals did not undergo to endoscopy and apparently they have regarded the individuals who do not show signs of illness, as asymptomatic. In current study, asymptomatic refers to those patients who underwent endoscopy but show no gastric abnormalities. However, this inconsistency in results may be related to differences of gene sequences affected by geographical and environmental variations (Salih, 2004).

Nomura *et al.,* (2002) by conducting a study to show the relation between the CagA antigen status and risk of peptic ulcers. As current study their results also showed that concentration of antigen is related to severity of diseases (figure 4-6).

In general, IL-17A, as its proinflammatory property, is elevated during inflammatory infection and anti-microbial responses (Onishi and Gaffen, 2010; Hasegawa *et al.,* 2017). Several studies conducted to reveal the role of IL-17A in gastric disease, but actually most of them investigated IL-17A at genetic (mRNA) level (Arachchi *et al.*; 2017). Khamri *et al.* (2010) showed the role of dendritic cells in the induction of CD4+ T cells to release IL-17A. They showed that *in vitro*, dendritic cells could profusely induce IL-17A secretion by skewing naïve CD4+ T cells differentiation to Th17 cells. They also exposed the role of *cagA* in the stimulation of IL-17A. Dendritic cells that incubated with *cagA* containing *H. pylori* strain were extremely potential producers than those

incubated with strain lacking *cagA*. Mizuno *et al.* (2005) conducted precise *in vitro* experiment to study IL-17A and IL-8 levels in normal and ulcer site of the antrum (in tissue not serum). They found a significant elevation of interleukins in patients infected with *H. pylori* than uninfected individuals and also significant elevation in ulcer site compared to tissue with normal appearance of infected patients. In their study all patients were infected with *cagA* positive *H. pylori*. In contrast to the results of current study (figure 4-9), their results did not show a significant differences between *H. pylori* positive gastric ulcer and *H. pylori* positive non ulcer patients. Another study that deals IL-17A and IL-8 levels *in vivo*, but also in mucosal tissue of infected patients, found significant differences between IL-17A levels of control and patients and CagA positive and CagA negative of both gastritis and peptic ulcer patients (El-Fakhry *et al.*, 2012). Arachchi *et al.* (2017) performed study dealt some interleukins at molecular and serum levels and showed that IL-17A serum levels differ in controls, *H. pylori* infected healthy individuals and infected patients. IL-17A levels were higher in *H. pylori* infected patients than *H. pylori* infected healthy individuals; and the latter have level higher than controls. However, the results of current study were aligned to those which reported presence of significant differences between all three groups of samples as shown in figures 4-7, 4-8 and 4-9. Actually, almost all studies indicate elevation levels of IL-17A in peptic inflammations.

In a study conducted to detect whether tTreg or Th17 cell is responsible for children infection by *H. pylori*, De Melo *et al.* (2012) found that IL-17A levels were higher in infected adults and children, but were significantly higher in former. Conversely, gastric levels of IL-10 were higher in children than adults of *H. pylori* positive groups. However, in contrast to this study, they found no correlation between incidence of

CagA and interleukins concentrations (figures 4-11 and 4-21; tables 4-4, 4-5). Luzza *et al.*, (2001) studied mRNA levels of different cytokines in the gastric mucosa of *H. pylori* infected children. Their study was aimed to understand the nature of immune response in the early phase of infection, represented by childhood, and identify whether Th1 or Th2 cytokines are implicated in host-pathogen interactions in this period. Overall the circumstances of their experiment do not resemble the current study, where it neither included adult individuals nor CagA producing *H. pylori* nor asymptomatic and symptomatic differentiation. However, they found that mRNA levels of IL-17A and IL-12, in relation to the current study, were elevated in *H. pylori* infected children, but IL-10 showed no relation to infection. Additional study performed by Serrano *et al.*, (2013) indicated that inflammatory responses in children occur in lower levels than adults as IL-17A, the pro-inflammatory cytokine, is higher in adults than in children. They reported identical mean levels of *H. pylori*, that been colonized the gastric mucosa of both children and adults. However, their study was at the molecular level (IL-17A mRNA) and IL-17A protein of gastric tissue. Nucleotide sequence analysis of *H. pylori* from all children and adults showed the same origin, which indicated that host factors instead of bacterial factors contribute to the reduce gastritis in children. In adults, IL-17A was higher in infected than uninfected and infected children but in contrast to the current study, they found no statistical significant at levels of IL-17A between uninfected and infected children compared to our results. However, in this study, serum levels of only symptomatic patients were shown (figure 4-11). Jafarzadeh *et al.*, (2009) in their published results showed that in patients with peptic ulcers serum level of IL-17A was significantly higher than asymptomatic patients. But in contrast to the current study while the serum level in asymptomatic was higher than uninfected controls there was no

significant difference between them (figures 4-7 and 4-8). The results of current study, as shown in figure 4-9 are similar to the results reported by De Melo *et al.* (2012) and Serrano *et al.*, (2013) and partially to Jafarzadeh *et al.*, (2009).

Haeberle *et al.*, (1997) published report indicated that alive *H. pylori* induce IL-12 production from Th1 significantly higher than killed *H. pylori*. De Jonge *et al.*,(2001) also demonstrated that *in vitro* Th1 incubated with killed bacteria would not produce IL-12 even the bacteria were *cagA* positive, and this result, intuitive as the arrival of CagA into the cells requires functional *cag* PAI. It has been improved that IL-12 is associated with the gastric infection caused by *H. pylori*. Bauditz *et al.*, (1999) published a report that showed significant elevation of tissue IL-12 in *H. pylori* positive chronic gastritis compared to *H. pylori* negative chronic gastritis, but in contrast to the results of current study there was no significant difference of IL-12 levels in patients infected with CagA positive and negative *H. pylori* (figure 4-13). Another study published by Sakai *et al.*, (2008) also showed that elevation of the IL-12 tissue level was higher in *H. pylori* positive patients than in *H. pylori* negative patients but the elevation was not significant. In patients with peptic ulcer disease also the mucosal level of IL-12 was higher compared to patients with non-ulcer diseases, but in contrast to the results of current study also without any significant level (figures 4-13 and 4-14). Luzza *et al.*, (2001) published research showed how gastric inflammation induced by *H. pylori* in children could be regulated by cytokines. Their study conditions were dissimilar to current study, because they compared cytokines levels between infected and uninfected children with *H. pylori* and did not dell the adults. Their study was at the gene level and indicated that IL-12 and IL-17A expression significantly increased in *H. pylori* infected children, but in contrast to this study IL-10 was not significantly elevated (figures

4-7,4-12 and 4-17). Additionally, another study published by Hida *et al.*, (1999) to investigate the expression of IL-12 and IL-10 mRNA in gastric mucosa infected with CagA positive *H. pylori*. In their study, all *H. pylori* infected patients were CagA positive. They found that mucosal IL-12 and IL-10 mRNA levels were significantly higher in CagA positive *H. pylori* patients than in CagA negative *H. pylori* (figures 4-12, 4-17).

To identify the association between serum IL-12 levels and CagA presence in *H. pylori* infected individuals, there was a study conducted by Eskandari-Nasab *et al.,* (2013) in Zabol, Iran. As in the current study, their study also included three types of samples: infected patients, asymptomatic carriers and healthy participants, but with some differences where asymptomatic and healthy individuals did not undergo to gastric endoscopy and the measuring of serum antibody instead of serum antigen. In their study, about half of asymptomatic samples were CagA negative. As current study, they found a significant elevation of IL-12 concentration in peptic ulcer with CagA positive *H. pylori* compared to asymptomatic and control and also significant elevation in asymptomatic compared to control. However, in their study, patients have not been distributed into age groups as here (figure 4-16). Augusto *et al.*, (2007) also indicated that mucosal expression of IL-12 was increased in patients infected with *H. pylori* and furthermore increased in patients with CagA positive *H. pylori.* However, in contrast to current study they did not take into consideration the asymptomatic group, *H. pylori* infected patients with normal gastric endoscopy finding (figures 4-12 and 4-13). De Melo *et al.*, (2014) published research had dealt finding the relation between age and some of cytokine concentrations in gastric mucosa to determine the type of immune response. Their results indicated high concentration of IL-12 in infected patients than in intact persons. The bacterial number in gastric tissue of adults and children was approximately equal, and there

was no difference in CagA positivity of *H. pylori* in adults and children. The results of current study were in line with their results concerning the concentration of IL-12 in children and adults, where current study also showed a decreased concentration of interleukin in children than in adults, and as their results in this study the concentration in children increased with age and in adults decreased with ageing (figures 4-12, 4-14 and 4-16).

As its role in limiting the span of activity of innate and the adaptive immune cells to support a homeostatic state, IL-10 found to be elevated in serum of recovered individuals from infection. However this increment is intended to avoid infection-associated immunopathology, allergy, and autoimmunity and so on (Ma *et al.,* 2015). Bodger *et al.*, (1997) published research to determine whether IL-10 plays role in gastric mucosal infected with *H. pylori*. In their study dyspeptic patients included CagA positive and negative *H. pylori* and there were also controls. They found that the IL-10 level was significantly increased in CagA positive *H. pylori* samples after 24 hours of *in vitro* incubation (figure 4-17), but there was no difference between CagA negative *H. pylori* acute and chronic gastritis and control. In study performed by Bauditz *et al.*, (1999) IL-10 level was higher in *H. pylori* infected patients than *H. pylori* negative patients with chronic gastritis and controls. In results of Bodger *et al.*, (1997) there was also no statistical significant in IL-10 level between CagA negative *H. pylori* patients with chronic gastritis and controls. However, in this study CagA negative *H. pylori* patients with chronic gastritis were excluded. Also in contrast to the results of current study there was no significant difference in IL-10 between CagA positive and negative *H. pylori* (figure 4-18). Serrano *et al.*, (2007) found that IL-12 and IL-10 levels changed with the severity of disease. All patients in their study enrolled to gastroduodenoscopy

were CagA positive and in the control group there were also *H. pylori* negative infected patients in addition to intact individuals. IL-12 and IL-10 were higher in *H. pylori* positive patients than in CagA negative *H. pylori* patients and control. Although interleukins were increased with the severity of disease, there was no association between the *cagA* and severity of disease, and this is contrary to what has been obtained in the current study (figures 4-12, 4-14, 4-17 and 4-19). In a study conducted to measure the levels of some cytokines in the mucosa and serum of *H. pylori* infected patients and controls, Abdollahi *et al.*, (2011) found that IL-10 neither different significantly between patients and controls nor between mucosal and serum level. Additionally, they found no association between IL-10, sex and age. All individuals in their study were underwent the gastroduodenoscopy. In current study, results showed some correlation between enrolled interleukins, sex and age (figures 4-17, 4-19, 4-20 and 4-21). In 2015, Michalkiewicz *et al.*, published a study dealt the investigation of some cytokines of the innate immune system in gastric mucosa of children infected with *cagA* positive *H. pylori* and *cagA* negative *H. pylori* at the mRNA levels. All samples were girls, age range 7-18, underwent gastric endoscopy and all of them did not peptic ulcers. While there was no difference in IL-12 levels among intact and infected children, IL-10 was significantly higher in infected than in controls, and among infected individuals those infected with *cagA* positive *H. pylori* were produced significantly higher IL-10 mRNA than those infected with *cagA* negative *H. pylori* (figures 4-12, 4-13, 4-17 and 4-18).

Helicobacter pylori virulence factors could induce many cytokines by the gastric epithelial cells. However, it should be kept in mind that *in vivo* there are complicated network of interactions which may occur, some of them are factors from other cells that infiltrate the injured gastric

mucosa (Pruett *et al.,* 2005). With regard to the current study, which aimed to understand how *H.pylori* can exploit its virulence factors to avoid immune system, CagA antigen was selected as one of the most important virulence factors, to study its probable role in the secretion of selected interleukins, IL-17A, IL-12 and IL-10, manipulation of the immune response and finally causing the transition of infection from acute to chronic phase.

Generally, upon bacterial infection, activated macrophages and dendritic cells rapidly produce IL-23 at the site of infection. Furthermore, *H. pylori*-NAP has been demonstrated to induce IL-23 secretion from macrophages and neutrophils (Amedei *et al.,* 2006). Apparently IL-23 production is *cagA* independent as its elevation in both asymptomatic and CagA positive *H. pylori* infected patients (Jafarzadeh *et al.,* 2009). IL-23 then by inducing differentiation and activation of local resident naive CD4+ T cells to Th17 and other IL-17A secreting cells, such as γδ and CD8+ T cells, initiate IL-17A secretion. It has been demonstrated that in gastric mucosa infected by *H.pylori* the major source of IL-17A is CD4+ T cells (Caruso *et al.,* 2007). IL-17A induces epithelial cells and lamina propria APCs to produce IL-8. In addition to its effect on IL-8 secretion, IL-17A offers other immune regulatory functions that cause *H .pylori*-related gastritis. For instance IL-17A provokes secretion of TNF-α, IL-1, IL-6 from both immune and non-immune cells such as monocytes, stromal and epithelial cells, and also stimulate fibroblasts to produce matrix metalloproteinase (MMPs), proteases family catalyzes cleavage multiple components in extracellular matrix, and so contribute in mucosal damages (Iwakura and Ishigame, 2006; Caruso *et al.,* 2007). Signaling pathways analysis IL-17A-induced IL-8 secretion indicated that in epithelial cell of *H. pylori* infected patients IL-17A activates ERK 1 / 2 MAP kinases and this pathway happens more severely in the case of

CagA positive *H. pylori* (Sebkova *et al.,* 2004). Also, IL-17A stimulates granulocyte-colony stimulating factor (G-CSF) by stromal cells; and therefore IL-23/IL-17A/ G-CSF pathway plays a crucial role in increasing neutrophils recruitment to the site of infection and bacterial clearance (Iwakura and Ishigame, 2006). GECs could be regarded as the primary site of contact between host and pathogen and they can behave in a double-acting manner, so they can express important inflammatory mediators and initiate the innate immune response and also present antigen to immune cells and promote adaptive immune system. During infection GECs produce IL-12 to increase the immune cells infiltration and recruitment to the site of infection (Al-Sammak *et al.*, 2013).

It is observed that during peptic infection, increasing production of IL-12 from Th cell is influenced by *cagA* positive strain of *H. pylori* (Hida *et al.,* 1999). Another study has revealed that complete functional *cag*PAI is required for production of IL-12 from APCs and not *cagA* alone (Galgani *et al.,* 2004). However, this issue is so far controversial (Eskandari-Nasab *et al.,* 2013). In response to bacterial products and after secretion from APCs, IL-12 plays an important role in the differentiation of naïve T cell into Th1 cells and production of IFN-γ and TNF- α. Subsequently IFN-γ stimulates phagocytic cells to secrete IL-12 which also induces IFN-γ production, creating a positive reinforcement loop (Parrello *et al.,* 2000). Some studies showed that during *H. pylori* infection, among APCs, monocyte/macrophage and neutrophils are responsible for IL-12 production instead of DCs (Kao *et al.,* 2006; Khamri *et al.,* 2010). Activated macrophages recruit neutrophils and the latter also produce IL-12 by themselves (D'Elios *et al.,* 2003). Production of IL-12 is pivotal in the development of Th1, which induces cell-mediated responses to infectious agents (O'Garra and Murphy, 1994). Several studies have pointed out that Th1 response is the signature of

gastric chronic inflammation developed by *H. pylori* infection (Bimczok *et al.*, 2011; Berenson *et al.*, 2004; Wang and Xu Landén, 2015), and this is a signal of initiation of adaptive immune response by bacteria (Pellicanò *et al.*, 2007). It has been shown that further production of IFN-γ by Th1 cells, lead to the development of chronic infection (Karttunen *et al.*, 1995). Actually together with IFN-γ, IL-12 dominate and increase the pathogenesis of *H. pylori* infection and finally cause mucosal damage and develop peptic ulcer rather than bacterial clearance (Crabtree, 1998; Deml *et al.*, 2005).

Helicobacter pylori has promoted a set of mechanisms to efficiently trick the adaptive immune system by intercepting of antigen presentation and modification of T cell responses. APCs simply engulf antigens by endocytosis or phagocytosis and by degrading and processing, offer them to CD4+ T cells via MHC class II molecules exhibited on cell membrane. This engagement initiates an antigen specific T cell response. In gastric mucosa of patient with *H. pylori*, increased numbers of activated APCs are noticed. Stimulated macrophages create IL-12 which provoke inflammation and encourage the Th1 responses (Peek *et al.*, 2010). Although of the effector cells recruitment, the successful persistence of *H. pylori* infection noted, suggesting the inability of these effector cells in clearance of the pathogen. It has also been shown that *H. pylori* polarize APCs such as macrophages to M1 subtype (Quiding-Järbrink *et al.*, 2010). The two main distinguished subsets of macrophages, M1 and M2, are differentiated from each other, whereas M1 (Previously it was referred to as activated macrophages) produces low levels of IL-10 and high levels of IL-12, M2 (alternatively activated macrophages) secrete high levels of IL-10 and low levels of IL-12 (Galdiero *et al.*, 2013).

Dendritic cell subsets also have been shown to promote T cell differentiation into antigen-specific Th17 cells, Th1 cells and IgA

producing B cells (Uematsu *et al.,* 2008; Denning *et al.,* 2011). Kao *et al.,* (2010) showed that DCs conducted by *H. pylori* block the Th17 response and will skew the response toward tTreg cell. They believe that this mechanism is depended on initiation of tTreg cells by IL-10 and TGF-β and independently on *H. pylori* virulence factors VacA and CagA. However Kaebisch *et al.,* (2014), showed that translocation of CagA into DCs inhibit host immune response by inhibiting the production of proinflammatory cytokines such as IL-12 and inducing the production of IL-10 as anti-inflammatory cytokine, indicating both inducing and inhibitory roles of CagA during infection depending on the cellular perspective. IL-10 suppresses functions of NK cells and T cells indirectly by preventing APCs from increased production of molecules implicated in antigen presentation, such as major histocompatibility complex II (MHC II) and lymphocyte activation and also from production of proinflammatory cytokines including IL-12 (Moore *et al.,* 2001). IL-10 also inhibits the increased expression of many genes in phagocytic and dendritic cells that are normally produced via TLRs stimulation of other PRRs. Although most effects of IL-10 are suppressive, they exerts some immune inducing effect such as initiation of cytotoxic T lymphocyte generation, B cell activation and upregulation of some of the genes in TLR activated phagocytic cells and DCs (Moore *et al.,* 2001). IL-18 and TGF-β produced by *H. pylori* activated DCs induce the development of tTreg cells, which subsequently produce and secrete IL-10 and block Th1 and Th17 responses (Dhar *et al.,* 2016). Involvement of tTreg cells in *H. pylori* infection was shown to be influenced by B7 family ligands and their related receptors, which are expressed on the surface of human epithelial cells, and represent important role in development and differentiation of T cells (Das *et al.,* 2006). B7 homolog 1 (B7-H1) is one of the major tTreg cell co-stimulatory factor in the initiation of

antigen-specific humoral and cell-mediated immune response (Dong *et al.*, 1999; Mitchell *et al.*, 2007). It has been shown that at the same time of T cell differentiation, GECs affected by *cag*PAI, up-regulate B7-H1 expression and this will cause deactivation of CD4+ effector T cell and up-regulation of tTreg cells (Larussa *et al.*, 2015). Furthermore, B7 family ligands are implicated in Th17 response. The effect of *H. pylori* and its major virulence factor CagA have been investigated in the modulation of B7-H2 in gastric mucosa. The *in vivo* and *in vitro* studies showed that CagA of *H. pylori* cause down regulation of B7-H2 on GECs, and inhibition the differentiation of CD4+ effector T cell into Th17 (Lina *et al.*, 2013). It has been shown that simultaneous suboptimal maturation of DC and decreased IL-12 secretion led to the inability of Th1 development and activation (Mitchell *et al.*, 2007). These studies indicated that *cagA* positive *H. pylori* also can indirectly stimulate IL-10 production and also can negatively regulate DCs function.

It has been shown that cytokines of both Th1 (e.g., IFN-γ) and Th2 (e.g., IL-10) are involved in positive and negative feedback regulation of IL-12 (Aste-Amezaga *et al.*, 1998). During *H. pylori* infection, IL-17A mediates the recruitment of neutrophils, a key cellular operator in the inflammatory lesion related to *H. pylori* infection (Fox and Wang, 2007). Expression of IL-12 and IL-23 genes in the stomach is also up-regulated and this reflects the stimulation of Th1 and Th17 cell responses, respectively (Khamri *et al.* 2010).

According to what has been stated it can be said that mixed Th17/Th1 cell response induced by *cagA* positive *H. pylori* may play important role in the gastric colonization and inflammation. However, in adults *H. pylori* caused gastritis is the resultant of Th17/Th1 immune mediated inflammatory pathways and in the gastric mucosa of infected children both are down regulated resulting in less severity of

inflammation and ulceration compared with adults (Serrano *et al.*, 2013) and this lead to the persistence of the infection in children (Bagheri *et al.*, 2015). However, in adults, there is increased secretion of IL-2 and IFN- γ which protect them from infection. IL-10 exerts its immune-regulatory function on Th1 cells to inhibit their responses and affects accessory cells to decrease IL-12 secretion and down regulate cell mediated responses (de Vries, 1995). More ever, IL-10 inhibits proinflammatory cytokines and chemokines secretion by macrophages and polymorphs, so potentially decrease neutrophil activation and blocks the reactive oxygen production, which cause tissue damage (Kasama *et al.*, 1994; Dang *et al.*, 2006).

In the current study, as mentioned in Figure (4-22) the ratio of inflammatory to anti-inflammatory interleukins secreted by the host in response to *H. pylori* also showed. The increased IL-10 ratio indicates entering of infection into chronic phase as IL-10 is represented as chronic infection related interleukin. However, this skewing is increased by influencing of CagA protein (Rudnicka *et al.*, 2013).

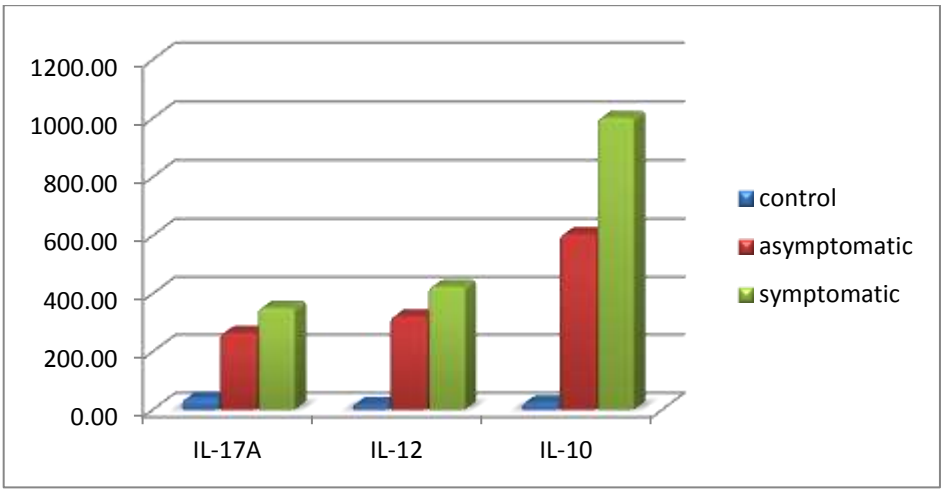

Figure 4-22: The levels of IL-17A, IL-12 and IL-10 in controls, asymptomatic and symptomatic patients.

However, there is no reason to indicate CagA role in production of IL-10 by tTreg cells. On the contrary, many studies deny the association between CagA protein and tTreg and even between CagA protein and Th2 inductions (figure 4-23). That is why it can be said that CagA induce cells other than tTreg and Th2, such as dendritic cells, B cells or macrophages (Larussa *et al.*, 2015; Mitchell *et al.*, 2012).

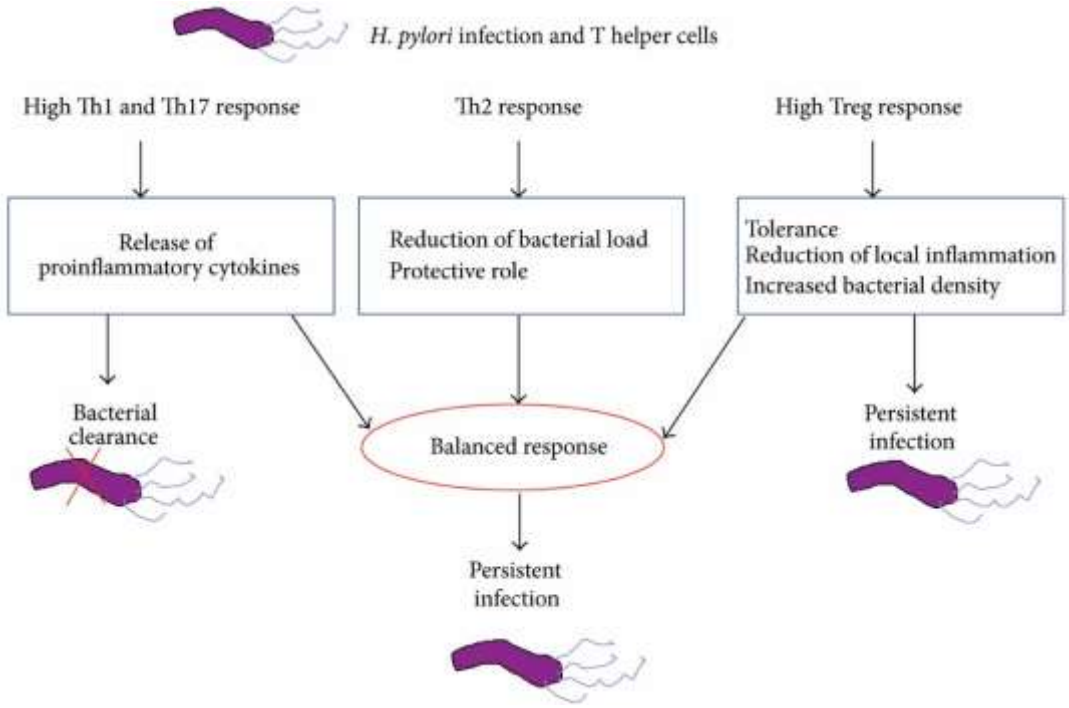

Figure 4-23: The interplay between *H. pylori* and the effective T helper lymphocytes. Although Th1 and Th17 pathways are both responsible for promoting inflammatory activities during *H. pylori* infection, neither Th1 nor Th17 cells are by themselves capable of a spontaneous clearance of the infection. It could be due to an impaired release of cytokines, suggesting that a more pronounced inflammation during the early phase of infection could switch the events towards eradication. Th2 response has been implicated in reducing bacterial load but its protective role is still controversial and deserves further investigation. tTreg cells limit local inflammation and tissue damage but at the same time this fact favours a tolerogenic status which leads to a persistent infection. This complex interplay suggests that the conflict between persistent infection and clearance is decided in the early phase of infection. (Larussa *et al.*, 2015).

Conclusions

1. There is a high correlation between gastroduodenoscopy finding and *cagA* gene and its product (CagA protein).
2. An association between the *cagA* gene and severity of peptic infection was found.
3. The interleukins (17A, 12 and 10) levels were significantly higher in symptomatic individuals (infected by Cag positive *H. pylori*) than asymptomatic individuals (infected by Cag Anegative *H. pylori*).

Recommendations

1- Performs *in vitro* can be studied the secretions of the interleukins (17A, 12 and 10) at different stages of the infection with *H. pylori* and the effect of antigen production on them.

2- A research can measure flow cytometry for CDs diagnosis in blood samples or biopsies

3- Measurement of the *cagA* gene in tissue and to compare between gene expression and protein circulation level (using CagA detection technique as used in the current study) to provide some confounding in the detection of anti-CagA antibody in serum of patients as the latter lead to false positive and negative results.

4- Both, ELISA antigen detection technique and ELISA antibody detection technique can be used to find whether of equilibrium between antigen and antibody concentrations, also to prove whether of any false positive or negative results.

5- Measurement the levels of studied interleukins in biopsies to find an association between their levels and histological distribution of cells probably secreting them, by using the same conditions of sample selection used in the current study, to determine which cells actually produced them.

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Appendix

Appendix

Questionnaire form

Assessment Questionnaire

H.Pylori RUT

Date 201 ..... /..... /..... No. : .....

Name ..... Gender..... Age .....

Address ..... Mobile ..... Work .....

Length ..... Weight .....

A. REASONS FOR GASTROSCOPY

☐ Surveillance (Family history of Gastric cancer/ Polyps) ☐ Smoking ☐ Alcohol ☐ Spices

☐ Symptoms: ☐ Weight loss ☐ Melena (upper GI bleeding) ☐ Anemia

☐ Epigastric pain ☐ Vomiting ☐ Obesity

B. CURRENT MEDICATIONS (Do you routinely take)

Antibiotic : ☐ Yes ☐ No

Proton Pump Inhibitor : ☐ Yes ☐ No

Blood Thinners: ☐ Yes ☐ No Aspirin Warfarin Heparin plavix 75mg Enoxaparin

NSAIDs: ☐ Yes ☐ No Voltaren (diclofenac) Ponstan (mefenamic acid) Indocid (Indometacin) Mobic (meloxicam)

C.MEDICAL HISTORY

1.Cardiovascular ☐ Yes ☐ No

☐ Valve Disease ☐ Cardiac Bypass ☐ Rheumatic Fever ☐ Hypertension

☐ Automatic Implantable Cardiac Defibrillator ☐ Ischemic Heart Disease (IHD)

2.Respiratory ☐ Yes ☐ No

☐ Shortness of Breath ☐ Home Oxygen ☐ Asthma ☐ T.B. ☐ Chest Infection

3.Others : ☐ Diabetic ☐ Renal Disease ☐ History of Constipation

☐ Infectious Disease (or recent exposure)

D. BIOPSY(BX.) DATE & NUMBER

OGD Finding :

E. PAST SURGERIES

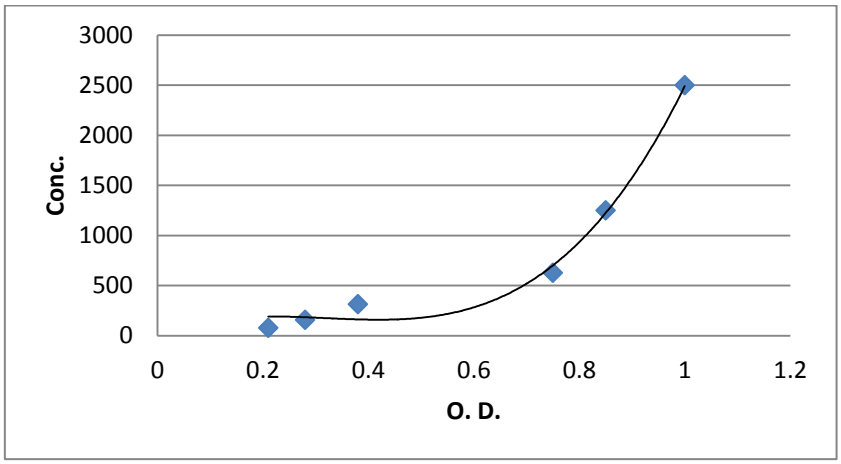
(Any Gastric or Duodenal surgery) ☐ Yes ☐ No

Type:

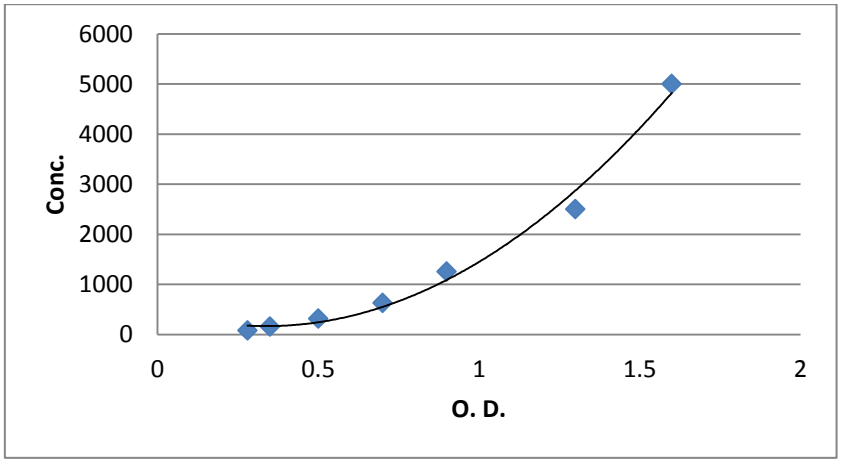
Drugs:

Appendix

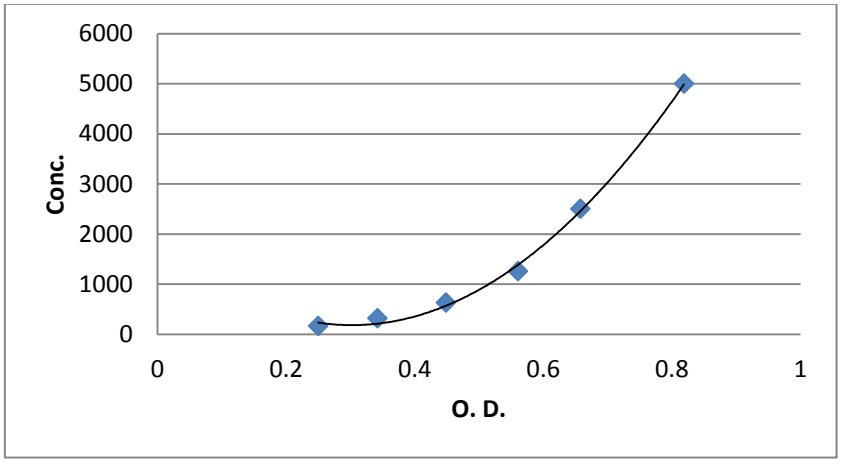
Interleukin 17A curve



Interleukin 12 curve

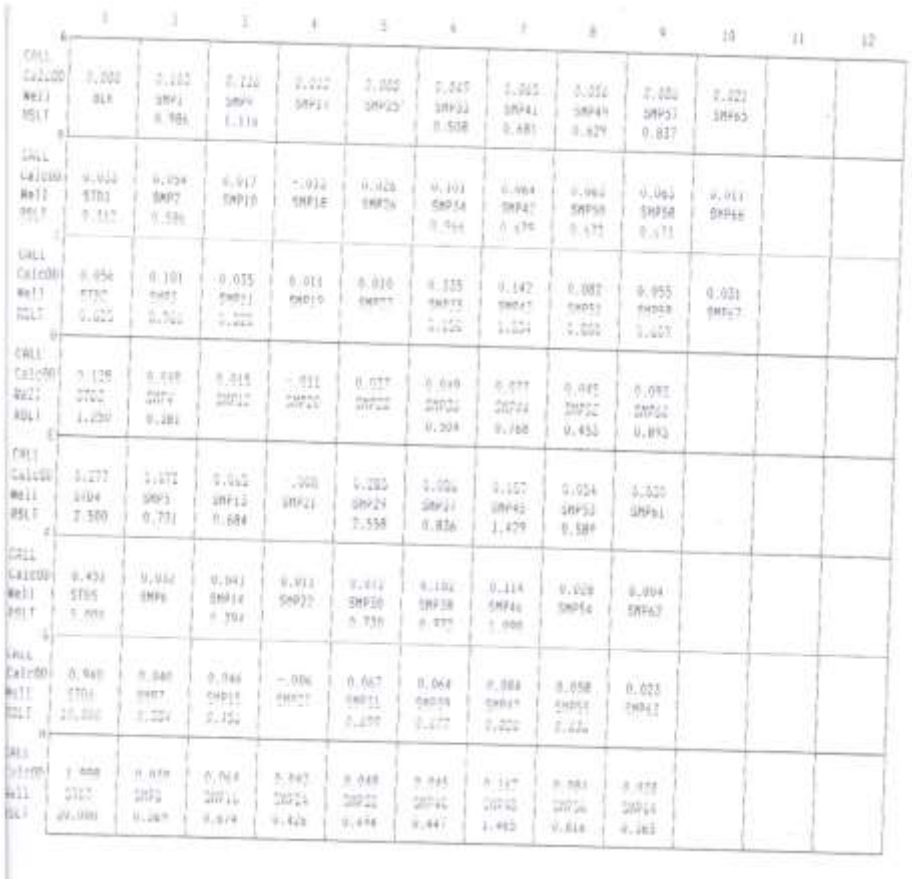


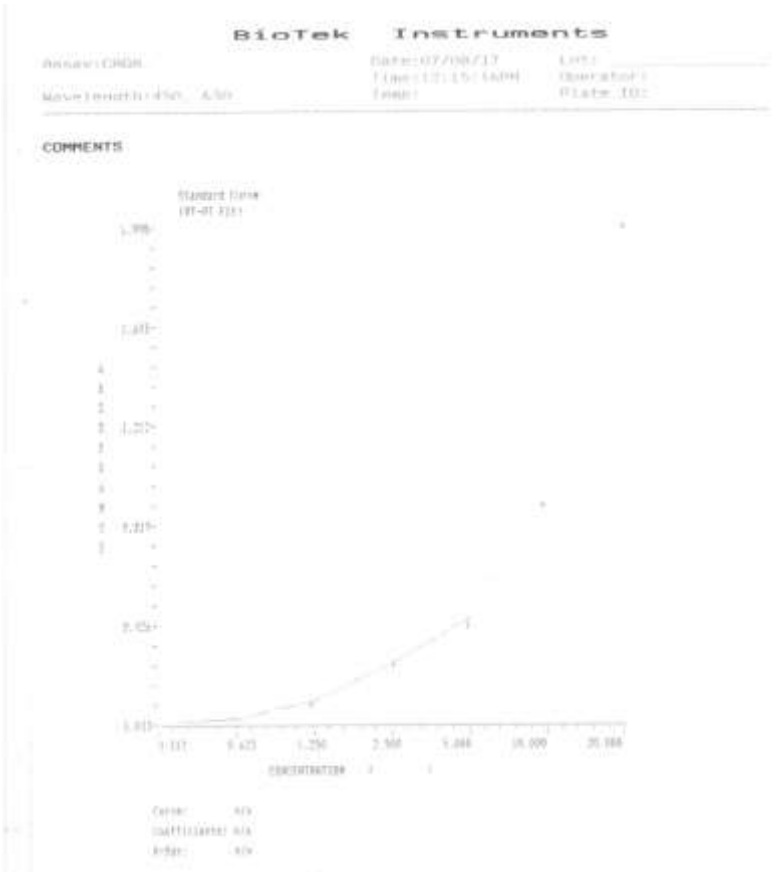
Interleukin 10 curve



Appendix

CagA data and curve





الملخص

أن مرض التهاب المعدة هو تهيج الغشاء المخاطي لبطانة المعدة. القرحة هي خراج مفتوح أو جرح للسطح الخارجي (الجلد) أو الداخلي (الغشاء المخاطي) للجسم وتتميز بالتهابات انسلاخية من الأنسجة الميتة أو فقدان الاسطح الظاهرية من الأنسجة.

كان الهدف من الدراسة هو معرفة كيفية هروب البكتريا اللولبية البوابية من الجهاز المناعي من خلال استخدامها للسم CagA و تأثيره على كل من الانترلوكينات 17، 12 و 10. تم جمع حوالي 245 عينة . تم استبعاد الاشخاص الذين تناولوا المضادات الحيوية و بعض العلاجات مثل الاميبرازول ، الاسبرين و المضادات غير الستيرودية. تم انتقاء 82 عينة من العدد المجموع و اللاتي تضمنت عينات خزع معدية و عينات دم من المرضى بمعدل عمر 32.54 و مدى 1 الى 60 سنة. تم اخضاع كل العينات للفحص الأولي, فحص اليوريز السريع, للتأكد من العينات سواء كانت مصابة ام لا. تضمن التشخيص الجزيئي للبكتريا اللولبية، استخدام الجين *glmM*. و من ثم تم استخدام الجين*cagA* للتحري عن العلاقة بين وجوده و الأعراض المرضية لدى المصابين بالبكتريا. تم قياس الانترلوكينات الثلاثة: الانترلوكين 17، 12 و 10.

تم توزيع المرضى الى ثلاث فئات حسب ما عثر عليه اثناء عملية التنظير العلوي و تضمنت اشخاص غير مصابين نسبتهم % 28.05 و اشخاص مرضى بدون اعراض 26.83% و اشخاص مرضى مع اعراض مرضية و نسبتهم كانت 45.12%. اضافة الى ذلك تم تصنيف المرضى ذوي الاعراض الى مرضى مصابين بإلتهاب المعدة (64.86%) و مرضى مصابين بالقرحة المعدية (35.14%). حوالي 94.59% من المرضى ذوي الاعراض قد اصيبوأ ببكتريا تحمل الجين*cagA* في حين 5.41% من المرضى ذوي الأعراض كانوا مصابين ببكتريا تفتقر الجين المذكور؛ و على العكس، المرضى الذين لم تظهر عليهم اعراض الاصابة في المعدة كانوا قد اصيبوا ببكتريا تحمل الجين *cagA* بنسبة4.55 % و المصابين ببكتريا تفتقر للجين كانت نسبتهم 95.45%. قياس مستوى المستضد CagA في المصل بين فروق معنوية بين الفئات المصابة عديمة الأعراض و الفئات المظهرة للأعراض ($p\leq0.0001$). وجدت علاقة قوية بين تركيز المستضد في المصل و شدة المرض.هناك فرق معنوي بين التهاب المعدة و القرحة ($p\leq0.001$).

كل الانترلوكينات المقاسة كانت ذات تركيز اعلى في المرضى من الاصحاء و بفروق معنوية كبيرة؛ و بالاضافة الى ذلك كانت نسبتها في الفئة المصابة المظهرة للأعراض معنوية اكثر من نسبتها في الفئة المصابة عديمة الأعراض. ايضا بينت القياسات علاقة عكسية بين

تراكيز الانترلوكينات IL-17 و IL-12 و IL-10 في الصغار والكبار. حيث كانت تراكيز IL-17 و IL-12 في الكبار اعلى من الصغار و على العكس كانت تراكيز IL-10 في الصغار اعلى من الكبار.

كما ظهر في المستضد كان هناك ارتباط عالي بين تراكيز الأنترلوكينات و الشدة المرضية في المصابين من التهاب المعدة الى القرحة المعدية.

بينت هذه الدراسة وجود علاقة وثيقة بين وجود الجين *cagA* و منتجه و وجود الاعراض المرضية في المعدة و الإثني عشر للمرضى العراقيين.

جمهوريـة العـراق
وزارة التعليم العالـي والبحث العلمـي
جامعـة كربلاء / كليـة العلـوم
قســم علــوم الحيـاة

بعض العلامات المناعية والجزيئية للقرحة المعدية

رســالة مقدمــة إلـــى

مجلـس كليـة العلـوم ــ جامعـة كربلاء

وهـي جـزء مـن متطلبـات نيـل درجـة الماجستير علــوم فــي علــوم الحيــاة

من قبل الطالب

محمد باقر شهيد عبدالزهرة الخطيب

(بكالوريوس علوم / علوم الحياة – 2009)

بإشراف:

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1439 هـ

2018 م