

ISTANBUL TECHNICAL UNIVERSITY * GRADUATE SCHOOL OF SCIENCE
ENGINEERING AND TECHNOLOGY

**MULTIPLEX-PCR BASED SCREENING AND COMPUTATIONAL
MODELING OF VIRULENCE FACTORS AND T CELL MEDIATED
IMMUNITY IN *HELICOBACTER PYLORI* INFECTIONS FOR ACCURATE
CLINICAL DIAGNOSIS**



Ph.D. THESIS

Sinem ÖKTEM OKULLU

Department of Molecular Biology-Genetics and Biotechnology

Molecular Biology-Genetics and Biotechnology Programme

JANUARY 2017

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**Sinem ÖKTEM OKULLU
(521092071)**

Department of Molecular Biology-Genetics and Biotechnology

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Thesis Advisor: Assoc. Prof. Dr. Ayça SAYI YAZGAN

Thesis Co-Advisor: Prof. Dr. Tanıl KOCAGÖZ

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

***HELICOBACTER PYLORI* ENFEKSİYONLARININ DOĞRU KLİNİK TANISI
İÇİN VİRÜLANS FAKTÖRLERİ İLE T HÜCREYE BAĞIMLI BAĞIŞIKLIĞIN
ÇOKLU-PZT İLE TARANMASI VE BİYOİNFORMATİK MODELLEMESİ**

DOKTORA TEZİ

**Sinem ÖKTEM OKULLU
(521092071)**

Moleküler Biyoloji-Genetik ve Biyoteknoloji Anabilim Dalı

Moleküler Biyoloji-Genetik ve Biyoteknoloji Programı

**Tez Danışmanı: Doç. Dr. Ayça SAYI YAZGAN
Tez Eş Danışmanı: Prof. Dr. Tanıl KOCAGÖZ**

OCAK 2017

Sinem Öktem Okullu, a PhD student of ITU Graduate School of Science, Engineering and Technology student ID 521092071, successfully defended the thesis entitled “MULTIPLEX-PCR BASED SCREENING AND COMPUTATIONAL MODELING OF VIRULENCE FACTORS AND T CELL MEDIATED IMMUNITY IN *HELICOBACTER PYLORI* INFECTIONS FOR ACCURATE CLINICAL DIAGNOSIS” which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

Thesis Advisor : **Assoc. Prof. Dr. Ayça SAYI YAZGAN**
Istanbul Technical University

Thesis Co-Advisor: **Prof. Dr. Tanıl KOCAGÖZ**
Acıbadem University

Jury Members: **Prof. Dr. Arzu KARABAY KORKMAZ**
Istanbul Technical University

Prof. Dr. Eda TAHİR TURANLI
Istanbul Technical University

Assoc. Prof. Dr. Işın AKYAR
Acıbadem University

Assist. Prof. Dr. Aşlı KUMBASAR
Istanbul Technical University

Assist. Prof. Dr. Necla Birgül İYİSON
Boğaziçi University

Date of Submission : 1 December 2016

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ABBREVIATIONS

µg	: Microgram
µl	: Microliter
µM	: Micromolar
µm	: Micrometer
ASA	: Acetylsalicylic Acid
BabA	: Blood Group Antigen-Binding Adhesion Protein A
bp	: Base pair
cagA	: Cytotoxin-associated gene A
Cag-T4SS	: Cag type IV secretion system
CCR-55	: Chemokine receptor type 5
CD	: Cluster of differentiation
cDNA	: Complementary DNA
CI_s	: Confidence Intervals
CO₂	: Carbon dioxide
Cp	: Crossing point
CTL	: Cytotoxic T lymphocyte
CXCR-3	: Chemokine (C-X-C) receptor 3
DC	: Dendritic cell
DMSO	: Dimethyl sulfoxide
DNA	: Deoxyribonucleic acid
dNTP	: Deoxyribonucleotide
dupA	: Duodenal ulcer promoting gene A
EIA	: Enzyme immunoassay
EDTA	: Ethylenediaminetetraacetic acid
ELISA	: <i>Enzyme</i> -linked immunosorbent assay
EPIYA	: <i>Glu-Pro-Ile-Tyr-Ala</i> motif
FOXP-3	: Forkhead box protein 3
FlaA/B	: Flagella A/B
FISH	: Fluorescence in situ hybridization
GATA-3	: GATA binding protein 3
GAPDH	: Glyceraldehyde 3-phosphate dehydrogenase
GI	: Gastrointestinal
H₂	: Hydrogen
HpaA	: Putative Neuraminylactose-binding Hemagglutinin Homolog A
<i>H. pylori</i>	: <i>Helicobacter pylori</i>
IFN-γ	: Interferon gamma
Ig	: Immunoglobulin
κ	: Cohen's kappa coefficient
IL	: Interleukin
Le^b	: Lewis b antigen
M	: Molar
MALT	: Mucosa-associated lymphoid tissue
Mg	: Milligram
MHC	: Major histocompatibility complex

Min	: Minute
mL	: Milliliter
m	: Middle region
mM	: Millimolar
mm	: Millimeter
mRNA	: Messenger ribonucleic acid
NADPH	: Nicotinamide adenine dinucleotide phosphate
NapA	: Neutrophil - Activating Protein A
NF-κB	: Nuclear Factor kappa B
NH₃	: Ammonia
NKT	: Natural killer T cell
NSAIDs	: Nonsteroidal anti-inflammatory drugs
OipA	: Outer membrane protein A
PAI	: Pathogenicity Island
PCR	: Polymerase chain reaction
pH	: Power of Hydrogen
PMN	: Polymorphonuclear leukocytes
PPI	: Proton-pump inhibitor
RGD	: Arginine-glycine-aspartate
RNA	: Ribonucleic acid
RUT	: Rapid urease test
RORγT	: RAR-related Orphan Receptor Gamma t
rRNA	: Ribosomal RNA
RT-PCR	: Real-time polymerase chain reaction
T-bet	: T-box transcription factor
T4SS	: Type IV secretion system
TCR	: T cell receptor
TGF-β	: Transforming Growth Factor-beta
Th1	: T helper 1
Th2	: T helper 2
Th17	: T helper 17
T_m	: Melting temperature
TLR	: Toll-Like Receptor
TNF	: Tumor necrosis factor
Treg	: Regulatory T cell
SAT	: Stool antigen test
s	: Signal region
STAT	: Signal Transducer and Activator of Transcription
UBT	: Urea breath test
UreA	: Urease A
UreB	: Urease B
<i>vacA</i>	: Vacuolating toxin gene A
qPCR	: Quantitative polymerase chain reaction

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MULTIPLEX-PCR BASED SCREENING AND COMPUTATIONAL MODELING OF VIRULENCE FACTORS AND T CELL MEDIATED IMMUNITY IN *HELICOBACTER PYLORI* INFECTIONS FOR ACCURATE CLINICAL DIAGNOSIS

SUMMARY

Helicobacter pylori (*H. pylori*) is a gram-negative, microaerophilic, spiral-shaped bacterium that colonizes the stomach of more than half of the world's population during their life span. The bacteria appear around 3µm in length and possess a unipolar bundle of two to six sheathed flagella, about 0,5µm in size, that enable the bacteria to move in the acidic environment of stomach. *H. pylori* infection is usually acquired in early childhood and persists throughout the life. While most infected individuals remain asymptomatic, *H. pylori* infection can cause peptic ulcer, chronic gastritis, mucosa-associated lymphoid tissue (MALT) lymphomas and gastric cancer in 20% of the infected ones. The prevalence of *H. pylori* is higher in developing countries and it may reach 70-80% of adults in Turkish population. While the exact route of transmission is unknown, acquisition may occur through fecal to oral or oral to oral contact. *H. pylori* infection can be diagnosed by the presence of dispeptic symptoms. Invasive and non-invasive methods are also used for the diagnosis of the *H. pylori* infection. Combination of antimicrobial agents and proton pump inhibitors has been used for the treatment of *H. pylori* infection; however, drug resistance may cause treatment failures, for which different drug combinations are used to overcome this problem. The outcome of *H. pylori* infection is closely related with bacteria's virulence factors, host genetic factors and host immune response. Bacterial virulence factors contribute to the inflammatory response towards *H. pylori* either by different mechanisms like host-signaling pathways, maintaining tissue homeostasis in epithelial cells or by stimulating the innate immune cells. Cytotoxin-associated gene A (*cagA*), vacuolating cytotoxin gene A (*vacA*), outer inflammatory protein A (*OipA*), blood group antigen-binding adhesion (*BabA*), putative neuraminylactose-binding hemagglutinin homolog A (*HpaA*), neutrophil activating protein A (*NapA*), duodenal ulcer promoting gene A (*dupA*), urease subunit A (*UreA*), urease subunit B (*UreB*) are the most important *H. pylori* virulence factors that play a role in the bacterial adhesion, colonization and infection.

Besides the virulence factors, type of the immune response of the host against *H. pylori* is crucial for the outcome of the infection. T cells are types of lymphocytes that play a central role in cell-mediated immunity against *H. pylori* infection. T cells develop in the thymus, and then enter the circulatory system. T cells are classified as cytotoxic T cells (CD8⁺ T cell), helper T cells (CD4⁺ T cell) and regulatory T cells (CD4⁺CD25⁺ T cell). Helper T cells differentiate into five major subsets known as T helper 1 (Th1), T helper 2 (Th2), T helper 17 (Th17), T helper 9 (Th9) and T helper 22 (Th22). T helper 1-based immune responses are required for protection against intracellular pathogens. T helper 2 cells are responsible for the protection against extracellular parasites. Recently described T helper 17 (Th17) cells are responsible

for mediating defensive mechanisms of the host against various infections including extracellular bacteria and fungi infections.

In previous studies, the association between T cells and *H. pylori* infection has already been identified. Th1 and Th17 cells are involved in response to *H. pylori* infection and contribute to the development of *H. pylori* – related pathology. Moreover, colonization with *H. pylori* leads to the expansion of regulatory T cells (Tregs) which limits the inflammatory response and cause the establishment of chronic infection. Th1, Th17, and regulatory T-cells (Treg) are localized in both gastric mucosa and peripheral blood of *H. pylori* – infected patients.

It is anticipated that there may be a close relationship between the *H. pylori* infection and bacterial virulence factors for the clinical outcomes of the infection. However, the effects of nine major *Helicobacter pylori* specific virulence factors; CagA, VacA, OipA, BabA, HpaA, NapA, DupA, UreA, UreB on Th1, Th17 and Treg cell response in *H. pylori* - infected patients with gastritis and ulcer have not been fully elucidated. Therefore in this study, we aimed to investigate the association between the nine major (CagA, VacA, OipA, BabA, HpaA, NapA, DupA, UreA, UreB) *Helicobacter pylori* specific virulence factors with three various T cell subsets (Th1, Th17 and Treg) at mRNA expression levels in *H. pylori*-infected gastritis and ulcer patients. The presence/absence of nine virulence factors were detected using multiplex-PCR and mRNA expression levels of T cell specific factors were determined using quantitative real time-PCR.

In total of 80 adult patients, 18 with ulcer and 62 with gastritis were included in this study. In addition, 18 patients with reactive gastropathy were included as the negative control group. These patients were admitted to Acibadem hospitals with gastrointestinal symptoms and underwent endoscopy. During endoscopy, two gastric antral and corpus biopsies were taken from each patient. One antral and one corpus samples were used for DNA isolation and the other antral and corpus samples were used for RNA isolation. Isolated DNA samples were used to develop a multiplex-PCR assay to detect nine *H. pylori*-specific virulence factor genes. Moreover, RNA samples were used to detect expression levels of Th1, Th17 and Treg cells - specific cytokines and transcription factors using quantitative real- time PCR (RT-PCR) assays. Primers were designed using primer design software for the *H. pylori* – specific virulence genes, Th1, Th17 and Treg cell – specific cytokines and transcription factors.

First step of the study was the development of multiplex-PCR to detect virulence factor genes. Optimization of multiplex-PCR assay was done by using *H. pylori* G27 strain that includes all the virulence genes. Also, a multiplex urease PCR assay was developed to detect *ureA* and *ureB* genes in a single PCR assay. In the second step, positive samples were detected by the conventional PCR for each cytokine and transcription factor. These positive samples were used to set up the standard curve in the quantitative real time-PCR for each cytokine and transcription factor.

Detection of the nine virulence genes using a multiplex-PCR assay within a three polymerase chain reaction was achieved; *ureA*, *ureB*, *cag A*, *hpaA*, *napA* in a one reaction, *oipA*, *dupA*, *vacA* in another reaction and *babA* in a third reaction. All DNA isolated from biopsy samples were tested with the developed multiplex urease PCR assay. A comparison study was performed between the results of the multiplex urease PCR assay, rapid urease test and pathological evaluation that were applied to patient samples. According to the results, it was determined that multiplex urease

PCR and pathological evaluation methods produced more similar and precise results than the rapid urease test method. Within all virulence factors that were tested, only the prevalence of NapA virulence factor was significantly higher in patients with ulcer than gastritis. Additionally, a positive correlation between the *H. pylori* DupA virulence factor and IFN- γ , and *H. pylori* BabA virulence factor and IL-17 was detected in gastritis and ulcer patients, respectively. By using computer-based models, clinical outcomes of patients infected with *H. pylori* can be predicted by screening the patient's *H.pylori vacA m1/m2*, *ureA* and *cagA* status and IFN- γ (Th1), IL-17 (Th17), and FOXP3 (Treg) mRNA expression levels. Herein, for the first time, the relationship between *H. pylori* virulence factors and host immune responses for diagnostic prediction of gastric diseases using computer-based models were reported. Furthermore, a novel expert - derived model has been developed to identify the set of factors and rules that may distinguish the ulcer patients from gastritis patients.





HELICOBACTER PYLORI ENFEKSİYONLARININ DOĞRU KLİNİK TANISI İÇİN VİRÜLANS FAKTÖRLERİ İLE T HÜCREYE BAĞIMLI BAĞIŞIKLIĞIN ÇOKLU-PZT İLE TARANMASI VE BİYOİNFORMATİK MODELLEMESİ

ÖZET

Helicobacter pylori dünya nüfusunun yarısından fazlasında, yaşamlarının bir döneminde görülen kronik mide enfeksiyonlarına yol açan bir bakteridir. *H. pylori* gram negatif, mikroaerofilik, spiral şeklinde bir bakteridir ve yaklaşık olarak 3 mikrometre uzunluğunda olup, 0.5 mikrometre çapında 2 ile 6 mikrometre arasında değişen hareket etmesine yardımcı olan kamçılara sahiptir. Çocukluk çağında kazanılan *H. pylori* enfeksiyonu ömür boyu sürmektedir. *H. pylori* ile enfekte olan bireylerin büyük çoğunluğu hastalık belirtisi göstermezken, bakterinin enfekte ettiği kişilerin % 20'sinde kronik gastrit, peptik ülser, mukoza ile ilişkili lenfoid doku lenfoması (MALT) ve mide adenokarsinomunda etiyolojik etken olduğu yönünde önemli bulgular vardır. Bakterinin prevalansı, gelişmekte olan ülkelerde gelişmiş ülkelere kıyas ile daha yüksektir. Ülkemizde ise erişkin toplumun yaklaşık % 70-80 kadarında bulunur. Bakterinin tam olarak bulaş yolu bilinmemekle beraber fekal-oral ya da oral-oral yollar ile bulaş olabildiği düşünülmektedir. *H. pylori* enfeksiyonunun tanısı genellikle dispeptik belirtilerin varlığı ve *H. pylori* enfeksiyonunu gösteren invaziv ve invaziv olmayan bazı testler yapılması sonucunda konur. Enfeksiyon tedavisi için kullanılan standart *H. pylori* antibiyotikleri ile proton pompası inhibitörlerinin yanı sıra son yıllarda hızla gelişen ilaç direnci *H. pylori* enfeksiyonunun tedavisi için farklı ilaç kombinasyonlarının uygulanmasını gerektirmiştir.

H. pylori enfeksiyonu sonucu oluşabilecek klinik tablolar bakterinin virülans faktörleri, konağın genetik faktörleri ve bağışıklık yanıtı ile yakın ilişki içerisindedir. *H. pylori*'nin virülans faktörleri bakterinin neden olduğu yangıya, doğal bağışıklık hücrelerini uyarmak, epitel hücrelerindeki doku homeostazisini korumak ya da konağın sinyal yollarını etkilemek gibi farklı mekanizmalar yardımı ile yol açmaktadır. *cagA* (sitotoksinle ilişkili A geni), *vacA* (vaküol oluşturucu sitotoksin geni), *babA* (epitel hücrelerde fukosillenmiş Lewis B antijenlerine bağlanan adezin proteinini kodlayan gen), *oipA* (dış zar enflamatuar A proteinini kodlayan gen), *hpaA* (hücreye bağlanmayı güçlendiren, hücre saldırısını kontrol eden adezin genleri), *ureA* ve *ureB* (üreaz enzimini kodlayan genler), *dupA* (duodenal ülser yönlendirici gen), *napA* (nötrofil aktive edici gen) *H. pylori*'nin mide mukozasına tutunmasında, kolonize olmasında ve mide de enfeksiyona neden olmasında etkili olan en önemli virülans faktörleridir.

H. pylori'nin virülans faktörlerinin yanı sıra konağın *H. pylori* enfeksiyonuna karşı oluşturduğu bağışıklık yanıtı da enfeksiyonun doğuracağı klinik tablolar açısından oldukça önemlidir. T hücreleri, hücresel bağışıklık yanıtının parçası olup *H. pylori* enfeksiyonundan korunmada önemli göreve sahiptirler. Gelişimleri timusta gerçekleşir ve sonrasında periferdeki sekonder lenfoid organlara göç ederler. T hücreleri, CD8⁺ sitotoksik T hücreleri, CD4⁺ yardımcı T hücreleri ve düzenleyici T

hücreleri (CD4⁺CD25⁺ Treg) olmak üzere üç gruba ayrılmaktadır. Yardımcı T hücreleri konağın gösterdiği bağışıklık yanıtında aracı hücrelerdir. Yardımcı T hücreleri T yardımcı 1 (Th1), T yardımcı 2 (Th2) ve T yardımcı 17 (Th17), T yardımcı 9 (Th9) ve T yardımcı 22 (Th22) olarak adlandırılan beş ayrı yardımcı T hücrelerine farklılaşmaktadır. Th1 hücreleri kaynaklı bağışıklık yanıtı hücre içi patojenlere karşı korumada rol almaktadır. Th2 hücreleri, hücre dışı parazitlere karşı oluşturulacak bağışıklık yanıtında görevlidir. Th1 ve Th2 hücrelerinden farklı olan ve yakın zaman önce tanımlanan Th17 hücreleri ise hücre dışı mikroorganizmalara karşı konak savunma mekanizmalarından sorumludur. Th9 hücrelerinin patolojik rolü çok açık olmamakla birlikte, pro-inflamatuvar Th17 hücrelerinin yol açmış olduğu bağışıklık yanıtını arttırdığı ve T hücre toleransını aracı olarak kullanarak allograft reddini önleyebildiği gösterilmiştir. Th22 hücreleri mikrobiyal patojenlere karşı savunmada, doku onarımı ve doku yenilenmesinde rol almaktadır.

Düzenleyici T hücrelerinin (Treg) temel görevi ise bağışıklık yanıtının kontrol altında tutulmasını sağlamaktır. Otoimmün yanıtın oluşmasının engellenmesinde ve enfeksiyon sonrasında patojen mikroorganizma temizliğinde de görev almaktadır. CD4⁺ T hücrelerinin polarize yanıtları, salgıladıkları sitokin profillerine göre farklılık göstermektedir.

Daha önce yapılan bilimsel çalışmalarda T hücreleri ile *Helikobakter pylori* enfeksiyonu arasındaki ilişki açıkça ortaya konmuştur ve belirtilen T hücre yanıtlarının *H. pylori*'nin farklı suşlarının içerdiği virülans faktörlerine göre farklılık gösterebileceği öne sürülmüştür. Konağın vücut bağışıklık yanıtına bağlı olarak, Th1 ve Th17 yardımcı hücrelerini kapsayan CD4⁺ T hücreleri *H. pylori* ile enfekte olan hastalarda enflamatuvar yanıtta yer almaktadır ve CD4⁺CD25⁺ düzenleyici T hücrelerinin miktarı *H. pylori* ile enfekte olan hastaların mide mukozasında ve periferik kanında yüksek oranda bulunmaktadır. Fakat *H. pylori* enfeksiyonunda önemli rolü olan 9 virülans faktörünün; CagA, VacA, OipA, BabA, HpaA, NapA, DupA, UreA, UreB *H. pylori* ile enfekte olan hastalarda görülen T hücre yanıtı ile (Th1, Th17 ve Treg) arasındaki ilişkileri tam olarak bilinmemektedir. Gerçekleştirdiğimiz çalışmamızda, *H. pylori* enfeksiyonunda önemli olduğu düşünülen dokuz virülans faktörü için geliştirilecek olan çoklu-PZT'nin sonuçları ile *H. pylori* ile enfekte gastritli ve ülserli hastalarda gelişen T hücre yanıtlarının, Th1, Th17 ve Treg, arasındaki ilişkinin belirlenmesi amaçlanmıştır.

Çalışmaya 1 yıl boyunca Acıbadem Hastaneleri Gastroenteroloji Servisi'ne dispepsi yakınmasıyla başvuran ve Endoskopi bölümünde gastroduodenoskopi uygulanan - 18-65 yaş arası, çalışma kriterlerine uygun 18 ülserli, 62 gastritli toplam 80 erişkin hasta dahil edilmiştir. Negatif kontrol olarak ise 18 reaktif gastropati tanısı konulan hasta kullanılmıştır. Endoskopi yapılan hastalardan antrum küçük kurvaturdan 2 adet, korpus büyük kurvaturdan 2 adet olmak üzere toplamda 4 adet biyopsi örneği alınmıştır. Alınan biyopsi örneklerinin birinden DNA, diğerinden ise RNA izolasyonu yapılmıştır. İzole edilen DNA örnekleri dokuz farklı virülans genini bir arada saptayabilmek için geliştirilmek istenen çoklu-PZT çalışmaları için kullanılmıştır. RNA örnekleri ise T hücre alt gruplarının belirteçleri olan transkripsiyon faktörleri ve sitokinlerin RNA düzeyinde ekspresyonlarının incelenmesi için kullanılmıştır. Virülans faktörleri ve Th1, Th17 ve Treg hücrelerine ait sitokinler ve transkripsiyon faktörlerini belirleyebilmek için primerler tasarlanmıştır. Çalışmaya virülans faktörlerini bir arada saptayabilmek için geliştirilmek istenen çoklu-PZT ile başlanılmış, *H. pylori*'ye ait olan tüm virülans faktörlerini taşıdığı bilinen G27 suşu pozitif kontrol olarak kullanılarak çoklu PZT

için tepkime denemeleri ve optimizasyon çalışmaları yapılmıştır. Ayrıca sadece *ureA* ve *ureB* genlerini tek bir PZT ile saptayabilen bir yöntemde geliştirilmiştir. T hücre alt gruplarının belirteçleri olan transkripsiyon faktörleri ve sitokinler için konvansiyonel PZT ile izlenebilir PZT’de standart eğriyi belirleyebilmek için pozitif hasta grupları bulunmuş ve optimizasyon çalışmaları tamamlanmıştır. Optimize edilmiş izlenebilir PZT ile içerisinde 62 gastritli ve 18 ülserli hasta grubunun bulunduğu 80 hasta için Th1, Th17 ve Treg hücrelerinin gruplarının belirteçleri olan transkripsiyon faktörleri (ROR γ T, FOXP3) ve sitokinlerin (IFN γ , IL-17) RNA düzeyinde ekspresyonları izlenebilir PZT ile incelenmiştir.

Çoklu-PZT optimizasyon çalışmaları sonucunda 9 farklı virülans genini üç farklı PZT karışımı ile saptamak olanaklı hale gelmiştir. *ureA*, *ureB*, *cag A*, *hpaA*, *napA* genlerinin bir karışımında, *oipA*, *dupA*, *vacA* genlerini bir karışımında ve *babA* genini bir karışımında saptamak olanaklı olmuştur. Çalışma sırasında geliştirilen yalnızca *ureA* ve *ureB* genini içeren çoklu bir PZT tepkimesi ile çalışma grubundaki hastalara üreaz taraması yapılmıştır. Geliştirilen çoklu üreaz PZT sonuçları ile hasta örneklerine uygulanan hızlı üreaz testi ve patolojik değerlendirme sonuçları arasında karşılaştırma çalışması yapılmıştır. Karşılaştırma çalışmaları sonuçlarına göre çoklu üreaz PZT ve patolojik değerlendirme yöntemlerinin hızlı üreaz test yöntemine göre daha benzer ve duyarlı sonuçlar verdiği belirlenmiştir. Çoklu-PZT sonuçları ve gerçek zamanlı-PZT sonuçları biyoinformatik çalışmalar ile bir araya getirilerek *H. pylori* enfeksiyonu sonucunda oluşabilecek gastrit ya da ülseri birbirinden ayırabilecek faktörleri birarada gösteren bilgisayar tabanlı modeller oluşturulmuştur. Çoklu-PZT sonuçlarına göre test edilen bütün virülans faktörleri içerisinde *napA* geni ülserli hastalarda gastritli hastalara göre daha yüksek oranda pozitif bulunmuştur. Ülserli ve gastritli hastalarda DupA virülans faktörü ile IFN- γ arasında, *babA* virülans faktörü ile IL-17 arasında pozitif ilişki bulunmuştur. Bilgisayar tabanlı modelleri doğrultusunda hastalardan izole edilen *H. pylori*’nin *vacA m1/m2*, *ureA* ve *cagA* gen durumlarının, enfekte olan hastaların IFN- γ (Th1), IL-17 (Th17) ve FOXP3 (Treg) ekspresyon seviyelerinin bilinmesi ile enfeksiyonun doğuracağı klinik sonuçları önceden tahmin etmek olanaklı hale gelmiştir. Yapılan bu çalışmada *H. pylori*’ye ait olan 9 virülans faktörü ile Th1, Th17 ve Treg hücre yanıtları arasındaki ilişki gastritli ve ülserli hastalarda açıkça ortaya konmuştur. Bu çalışma ile literatürde bilindiği üzere ilk defa *H. pylori* ile enfekte olan hastalardan izole edilen bakteri izolatlarının sahip olduğu virülans faktörleri ile hastaların bağışıklık yanıtı ilişkilendirilerek bilgisayar bazlı modeller kullanarak klinik sonuçlarını önceden tahmin etmek olanaklı hale gelmiştir.



1. INTRODUCTION

1.1 Human Stomach

Helicobacter pylori (*H. pylori*) has its ecological niche in the human stomach which is a thick-walled muscular organ that lies between the esophagus and the first part of the small intestine. Together with the digestive process function, human stomach eliminates the invasion of microorganisms because of its highly acidic environment where pH range changes between 1 and 2, due to the gastric acid production. In addition, the entire epithelial surface of the human stomach is covered with a thick layer of secreted mucus that acts as a physical barrier to prevent pathogens from colonizing and interacting with the underlying epithelium (Figure 1.1).

However, *H. pylori* is able to colonize in the acidic environment of stomach by overcoming the thick mucus barrier. Urease enzyme production and motility function of *H. pylori* are two essential factors that play role in colonization inside the human stomach (Sherwood, 1997).

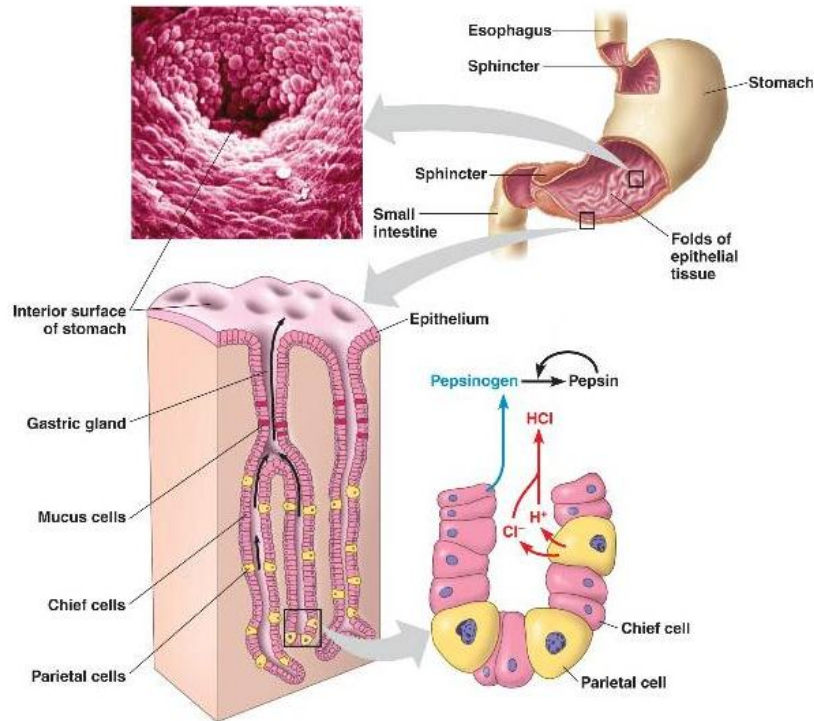


Figure 1.1 : Structure of human stomach (adapted from Cancer Information and Support Network [CISN], 2013).

1.2 Microbiology of *H. pylori*

1.2.1 Morphology

H. pylori is a gram negative, spiral - shaped bacterium, approximately 3.5 μm in length and a diameter of about 0.5 μm . It is motile normally with a unipolar bundle of two to six sheathed flagella (about 3 μm long) which enable the bacteria to move in mucous layer of the gastric epithelium (Figure 1.2) (Doig *et al.*, 1995). Each flagellum has a flagellar sheath as an extension of the outer membrane that protects the acid-labile flagellar structure from the attack of the stomach acid (Hawtin *et al.*, 1990).

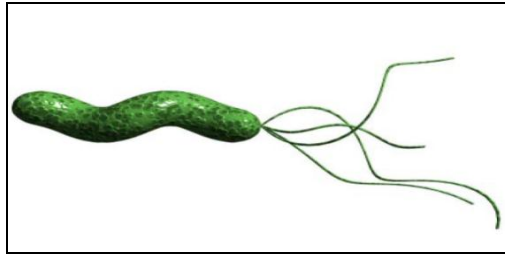


Figure 1.2 : Image of *H. pylori*, 10,000x computer- aided design, shows curved shape and flagella of the bacterium that enable the bacterium to propel itself into the mucus lining of the stomach (adapted from The *Helicobacter* Foundation, 2015).

1.2.2 Cellular envelope

H. pylori has a unique outer membrane structure. The peptidoglycan layer of *H. pylori* contains a high amount of mucopeptides, with a pentapeptide side chain ending in glycine and containing (1–6)-anhydro-N-acetylmuramic acid (Costa *et al.*, 1999).

1.2.3 Respiration and metabolism

H. pylori is a microaerophilic bacterium that requires oxygen, but at a lower concentration than found in the atmosphere. *H. pylori* is a slow growing microorganism that can be cultured on solid media including blood or selective media like brucella agar supplemented with 5% horse blood, or brain heart infusion media with 7 % horse blood incubated at 37°C for 3 to 7 days under 5% to 10% oxygen and 5% to 12% carbon dioxide with humidity conditions (Goodwin & Worsley, 1993).

H. pylori produces oxidase, catalase, and urease enzymes that facilitate biochemical diagnosis of bacteria (Stark *et al.*, 1999). Urease enzyme is vital for bacterial survival and colonization in the acidic stomach environment. In addition, bacterium contains a hydrogenase, which can be used to obtain energy by oxidizing molecular hydrogen (H₂) produced by intestinal bacteria (Olson & Maier, 2002). *H. pylori* species are capable of forming biofilms (Stark *et al.*, 1999). When the environment is not suitable for its survival it can convert from spiral to viable but nonculturable coccoid form.

1.2.4 Microscopic features

Under the microscope, it is possible to detect *H. pylori* that is stained by gram stain, giemsa stain, haematoxylin-eosin stain, Warthin-Starry silver stain and acridine-orange

stain. When the bacteria are stained with the gram staining it appears as gram-negative rods (Bode *et al.*, 1993).

1.3 History of *H. pylori* Infection

Over 100 years ago, a Polish Clinical Researcher Prof. W. Jawroski at the Krakow Jagiellonian University described spiral shaped microorganisms in the human stomach. In the late 1970s Robin Warren, a Pathologist in Perth, noticed these bacteria on the inflamed gastric mucosa. He wondered whether there was a relationship between the inflammation and these bacteria. Then he learned that European pathologists had found similar microorganisms in the late 19th century, but they were not able to grow them in culture (Marshall & Warren, 1984). Barry Marshall, a young trainee in internal medicine, started to work with Warren on his observations. However, initially they had also difficulties culturing these unknown bacteria. They thought this curved shaped gram-negative bacteria from the biopsy samples were *Campylobacter* species, which cultured on selective media under microaerobic conditions within 48 hours. They began with 30 patients, but they did not achieve cultivation of the microorganism isolated from biopsy samples. Then, the Easter Holidays arrived; accidentally one of the cultures was incubated 5 days instead of the usual 2 days in the hospital laboratory and colonies were seen on this culture plate. Microorganisms isolated from the patients were called as *Campylobacter* like organisms (CLO) then in a short period of time *Campylobacter pyloridis* and *Campylobacter pylori* because of their similarity to the *Campylobacter* genus. Later, based on the differences between the newly isolated bacterium and *Campylobacter*, new bacterium was called as *Helicobacter pylori* (*H. pylori*) (Marshall & Warren, 1984). In 1985, Barry Marshall swallowed *H. pylori* to prove that it causes ulcer. However, instead of developing ulcer symptoms, he developed gastritis symptoms. In this way, Barry Marshall proved that *Helicobacter pylori* caused gastritis. At this time, it had become clear that *H. pylori* infection was strongly associated with gastric mucosa inflammation. Later connection between *H. pylori* and ulcer was eventually deduced from epidemiological studies that showed an increased incidence of ulcers in persons infected with the bacteria.

After Warren and Marshall's publication, other investigators were able to culture the bacteria from their patients with gastritis and ulcer. In 1994, National Institutes of Health in America concluded that *H. pylori* was a major cause of peptic ulcer diseases as noted earlier by Marshall and Warren. In 2005, Warren and Marshall were awarded the Nobel Prize for their work on *H. pylori* (Kirsner *et al.*, 1994).

1.4 Epidemiology of *H. pylori* Infection

Over the last few decades, the epidemiology of *H. pylori* infection has been changing in most countries with a decline of the prevalence of the infection. Changes have been confederate with a paralel decline in peptic ulcer disease, gastric cancer and other related diseases as gastroesophageal reflux disease, allergies and asthma. However, it has been shown in several studies that the prevalence of *H. pylori* is still high in most countries. In north European and North American populations, about one-third of adults are still infected with this pathogen, whereas in south and east Europe, South America, and Asia, the prevalence of *H. pylori* infection is frequently higher than 50% (Eusebi *et al.*, 2014; Sonnenberg, 2013).

1.4.1 Prevalence of *H. pylori* infection

The global prevalence of *H. pylori* infection is more than 50%. In developing countries the prevalence is higher than in the developed countries (Table 1.1 and 1.2). The prevalence of *H. pylori* infection is closely related with age, ethnicity, gender, geography and socioeconomic status (Sonnenberg, 2013).

In Europe, the prevalence of *H. pylori* infection appears to be lower in Northern countries than in Eastern and Southern countries (van Blankenstein *et al.*, 2013). In South-Western Europe, Portugal, the prevalence reaches to 84.2 %. Turkey has the highest prevalence of *H. pylori* infection among the eastern European countries (Ozaydin *et al.*, 2013). In North America, the prevalence of *H.pylori* seems to be similar to northern Europe (Sethi *et al.*, 2013). In Asia, prevalence of *H. pylori* infection changes from 28.3 % to 86 %. Nigeria has the highest prevalence rate between the countries of Africa (Fock & Ang, 2010).

The prevalence of *H. pylori* infection in children is shown in Table 1.2. In Portugal the prevalence rate is 66.2%. In Brazil prevalence rate is 41.1% among children and young adults aged between 2 and 19 years old. Higher rate of infection was reported in Iran, where the prevalence is 50.5% (Ghasemi-Kebria *et al.*, 2013).

Table 1.1 : Prevalence of *H. pylori* infection in adults reported by studies published in 2013 (Eusebi *et al.*, 2014).

Country (Reference)	Setting	Number	Diagnostic Method	Prevalence of <i>Helicobacter pylori</i> %	References
Western Europe					
The Netherlands	Blood donors	1550	Serology	31.7	(van Blankenstein <i>et al.</i> , 2013)
The Netherlands	Pregnant women	6837	Serology	46	(den Hollander <i>et al.</i> , 2013)
Portugal	General population	2067	Serology	84.2	(Bastos, Peleteiro, Barros, <i>et al.</i> , 2013)
Eastern Europe					
Cyprus	Patients with dyspepsia	103	PCR	39.8	(Krashias, Bashiardes, Potamitou, Potamitis, & Christodoulou, 2013)
Turkey	General population	4622	UBT	82.5	(Ozaydin <i>et al.</i> , 2013)
America					
Canada	Aboriginal population	203	Histology	37.9	(Sethi <i>et al.</i> , 2013)
Mexico	Pregnant women	343	Serology	52.2	(Alvarado-Esquivel, 2013)
Asia					
Saudi Arabia	Healty individuals	456	Serology	28.3	(Sethi <i>et al.</i> , 2013)
Korea	Routine health check-up	10796	Serology	54.4	(Fock & Ang, 2010)
India	Patients with dyspepsia	2000	Histology RUT	58	(Sodhi <i>et al.</i> , 2013)
India	Patients with dyspepsia	530	Histology Urease Test	62	(Adeleka S, 2013)
China	Healty individuals	5417	UBT	63.4	(Y. Zhu, Zhou, Wu, Su, & Zhang, 2014)
Bhutan	Volunteers	372	Histology RUT Culture Serology	73.4	(Vilaichone <i>et al.</i> , 2013)
Bhutun	Patients with dyspepsia	244	Serology	86	(Dorji <i>et al.</i> , 2014)
Kazakhstan	Asymptomatic and patients with dyspepsias	835	Serology	76.5	(Benberin, 2013)
Africa					
Ethiopa	Selected population	1388	Serology	65.7	(Mathewos, 2013)
Morocca	Patients with dyspepsia	429	Histology RUT Culture	75.5	(Benajah, 2013)
Nigeria	Patients with dyspepsia	125	Serology Histology	93.6 80	(Benajah, 2013)

Table 1.2 : Prevalence of *H. pylori* infection in children reported by studies published in 2013 (Eusebi *et al.*, 2014).

Country (Reference)	Age of included subjects	Number	Diagnostic method	Prevalance of <i>Helicobacter pylori</i> %	References
Western Europe					
Belgium	12-25	516	UBT	11	(Olokoba, Gashau, Bwala, Adamu, & Salawu, 2013)
Portugal	13	1312	Serology	66.2	(Bastos, Peleteiro, Pinto, et al., 2013)
America					
Brazil	2-19	129	Histology RUT Culture	41.1	(Bastos, Peleteiro, Pinto, et al., 2013)
Asia					
China	1-18	1634	Histology RUT	32.1	(Yu, Su, Wang, Wang, & Xu, 2014)
Iran	1-15	194	Serology	50.5	(Ghasemi-Kebria et al., 2013)

1.4.2 Risk factors for *H. pylori* infection

Risk factors for *H. pylori* infection appears to be during childhood and early adult years (Gersten & Wilmoth, 2002). The vast majority of infections are asymptomatic, but infected individuals often have histological evidence of gastritis. Household hygiene, socioeconomic status, occupation, family income level, living conditions, cultural background and genetic factors are mainly focused risk factors for *H. pylori* infection. Individuals with lower education have higher risk than individuals with higher education. Also, the parent's education level is one of the determinants as parents with higher education have lower risk for the *H. pylori* infection. Although no significant association has been found between the lifestyle habits, smoking, dietary, alcohol drinking, gender, age, and infection, these factors may be supposed as risk factors for *H. pylori* infection (Dorji *et al.*, 2014; Hanafi & Mohamed, 2013; Vilaichone *et al.*, 2013).

1.4.3 Transmission

The mode of transmission of *H. pylori* infection remains poorly understood. Although person-to-person transmission is accepted as the main route, there are many other transmission routes (Akamatsu *et al.*, 1996). Iatrogenic transmission occurs when the same tubes or endoscopes are used for different patients during endoscopy (Lin *et al.*, 1994). Consumption of faeces contaminated water or foods are examples for the fecal to oral transmission route. The use of the same spoon by both mother and child is one of the causes for the oral-to-oral transmission route of *H. pylori* infection (Megraud, 1995).

1.4.4 *H. pylori* recurrence after eradication treatment

Recurrence of *H. pylori* infection after successful eradication treatment is rare in developed countries and more frequent in developing countries (Gisbert, 2005). Recurrence is defined as recolonization of the same strain rather than a new strain that is defined as reinfection. As a clinical problem, recrudescence develops as a result of the treatment failure. Recrudescence rate is very high during the first year of treatment, then this rate decreases. However, reinfection risk is high for the treated patients during their lifespan. For *H. pylori* infection accurate diagnosis is difficult and most diagnoses rely on molecular methods which confirm that the identified bacteria are genetically identical (Peitz *et al.*, 1999; Xia *et al.*, 1997). High *H. pylori* recurrence rate has been seen in areas with low socioeconomic development because of the failure in treatment.

1.5 *H. pylori* Associated Diseases

Since the initial report about *H. pylori* by Marshall and Warren, it remains one of the most common human infections which are associated with a number of important upper gastrointestinal (GI) diseases including chronic gastritis, peptic ulcer disease, gastric cancer, and mucosa-associated lymphoid tissue lymphoma.

1.5.1 Gastritis

Gastritis is defined as the inflammation of the stomach in which peptic ulcer and gastric cancer may develop (Moller *et al.*, 1994). The relationship between gastritis and *H. pylori* infection can be defined as the adhesion of *H. pylori* to the stomach lining and colonization inhibit the secretory functions of the mucus lining of the stomach and indicate a potential deleterious effect on this primary defense mechanism of the gastric mucosa. A sudden and severe stomach inflammation is called as acute gastritis. When inflammation endures for a long time that is called chronic gastritis. Erosive gastritis is another type of gastritis that often does not cause significant inflammation but can erode the stomach lining (Dixon, 2001).

1.5.2 Peptic ulcer

Peptic ulcer is a defect in the gastrointestinal mucosa when protective factors are weakened or the mucosa is exposed to large amounts of aggressive luminal factors, such

as acid and pepsin. One of the main causes of peptic ulcer is *H. pylori* infection. Colonization of *H. pylori* leads to increase in releasing level of gastrin and higher amount of gastrin promotes increase in acid secretion which damages intestinal lining causing peptic ulcer. Nonsteroidal anti-inflammatory drugs (NSAIDs) and acetylsalicylic acid (ASA) drugs are accepted as cause of at least 25% of the peptic ulcers (Bao *et al.*, 2010; Wu *et al.*, 2009).

1.5.3 Gastric cancer

In 2012, approximately 1 million cases of gastric cancer are diagnosed in the world (952,000 cases, 6.8% of the total) making the gastric cancer fifth most common malignancy. Gastric cancer is the third leading cause of death in cancer for both sexes' worldwide (723,000 deaths which make up 8.8% of the total) (IARC, 2012).

The World Health Organisation's Agency Research for Cancer defined the *H. pylori* as a carcinogen in 1994 (Moller *et al.*, 1994). *H. pylori* infection is the main cause of gastric cancer. In addition to *H. pylori* infection and accompanied inflammatory response there are other risk factors for gastric cancer such as host gene polymorphisms and dietary factors like high salted diet, older age, gender, tobacco smoking, a history of stomach surgery for benign conditions, and a family history of stomach cancer (Marshall *et al.*, 1985).

Diffuse-type gastric cancer and intestinal-type adenocarcinoma are two distinct variants of gastric carcinoma. Diffuse-type gastric cancer is defined as infiltration of neoplastic cells that do not form glandular structures. Intestinal-type adenocarcinoma is defined as the transition from normal mucosa to chronic superficial gastritis followed by atrophic gastritis and intestinal metaplasia resulting with dysplasia and adenocarcinoma (Correa, 1996; Sipponen & Marshall, 2000).

Mucosa-associated lymphoid tissue (MALT) lymphoma is another cancer type associated with chronic *H. pylori* inflammation. Untreated *H. pylori* infection causes inflammation of the stomach lining and over time, this may lead to MALT lymphoma development (Parsonnet *et al.*, 1994).

1.6 Pathogenesis of *H. pylori* Infection

The pathogenesis of *H. pylori* infection in humans begins with entry; adherence and colonization of the bacteria to human gastric mucosa then continue with avoidance, subversion, or exploitation of the bacteria from the human immune system. This lasts with multiplication, tissue damage, and transmission to a new susceptible host or spread to adjacent tissue. Mainly, persistence and pathogenesis of *H. pylori* in the human stomach depends on its colonization on the gastric mucus layer and bacterial virulence factors which play important role in the progress of infection.

1.6.1 *H. pylori* colonization

The colonization of the *H. pylori* on the gastric mucus layer is the basis for pathogenicity and virulence (Figure 1.3). Motility is the first crucial step in the colonization of *H. pylori*. Flagella are essential for the motility of *H. pylori* and are important for the early stage of infection. *H. pylori* presents a typical bulb-like structure at its distal end that corresponds a dilation of the flagellar sheath. The sheath of flagella is an extension of the bacterial outer membrane and it protects the acid-labile flagellar structure from the attack of the gastric acid (Geis *et al.*, 1993).

There are three structural elements of the *H. pylori* flagella: a basal body that is embedded in the cell wall and comprises the proteins needed for rotation and chemotaxis; an external helically shaped filament that works like a propeller when rotated at its base; and a hook that attends as a joint between the flagellar filament and the basal body. Approximately there are more than 50 putative proteins that are involved in expression, secretion, and assembly of flagellar apparatus (Alm *et al.*, 1999; Tomb *et al.*, 1997). FlaA and FlaB are two flagellar proteins that play a role in the full motility of bacteria. FlgE and FliD are hook-associated proteins that are also essential for the motility and colonization (O'Toole *et al.*, 1994).

The second crucial step in the colonization is urease enzyme production of *H. pylori*. By the help of the urease enzyme bacteria can live in the acidic conditions of the gastric lumen. In the absence of urea, *H. pylori* can survive at a pH range between 4.0 and 8.0. However, urea helps *H. pylori* to survive at pH lower than 2.5. Urease enzyme catalyses the hydrolysis of urea to carbon dioxide (CO₂) and ammonia (NH₃). Production of NH₃,

which acts as an acceptor for the H^+ ions, leads to increase of local pH of the stomach environment where *H. pylori* can colonize. In addition, the production of NH_3 may facilitate the formation of NH_3 -derived compounds, such as monochloramine, which has cytotoxic effects on host cells (Suzuki *et al.*, 1992). Urease production of *H. pylori* allows short-term survival in the highly acidic environment of gastric lumen, whereas motility allows rapid movement toward the more neutral pH zone of the gastric mucosa (Nakamura *et al.*, 1998).

Adherence is the third crucial factor for *H. pylori* colonization in the human stomach. Adhesion to the gastric epithelium prevents the bacteria being eliminated from the stomach by mucus turnover and gastric peristalsis. In addition, it helps to deliver bacterial effector proteins such as CagA and VacA to the host cell and has an important role in the nutrient uptake of bacteria from the host cells, which were damaged by the inflammation. Better adherence means better colonization in the host gastric epithelium. *H. pylori* expresses different adhesins that bind to specific host-cell receptors, to facilitate adherence on host tissue surface (Testerman *et al.*, 2001).

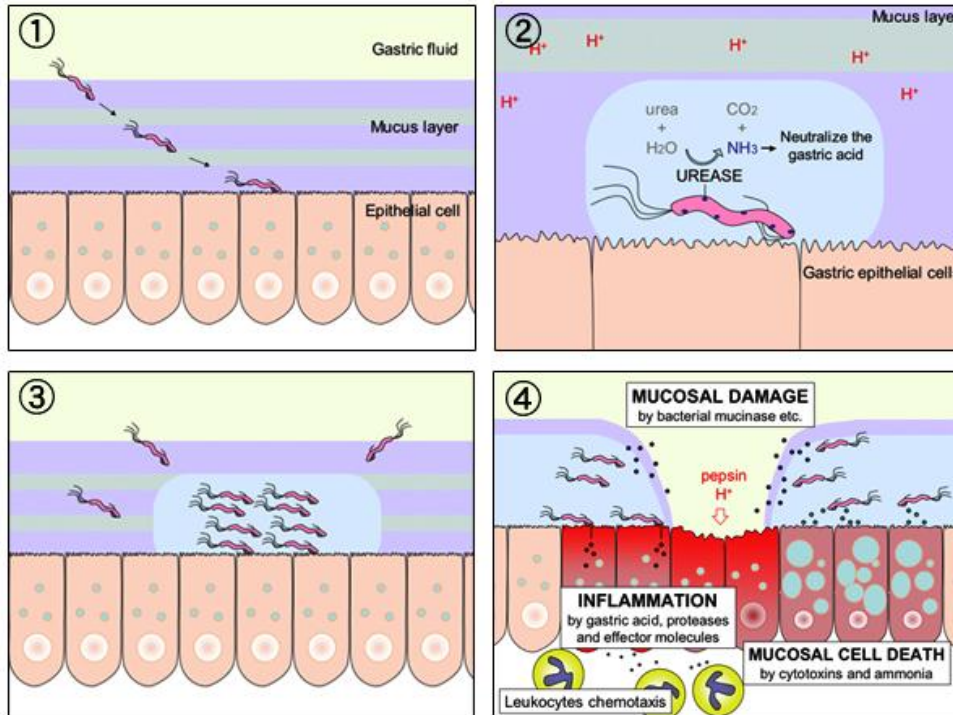


Figure 1.3 : The colonization of the *H. pylori* on the gastric mucus layer; (1) *H. pylori* penetrate the mucus layer of host stomach and adhere the surface of gastric mucosal epithelial cells, (2) produce ammonia from urea by the urease, and the ammonia neutralizes the gastric acid to escape from elimination, (3) proliferate, migrate, and finally form the infectious focus, (4) the gastric ulceration is developed by destruction of mucosa, inflammation and mucosal cell death (adapted from BioWeb.edu.tr, 2016).

1.6.2 Virulence factors of *H. pylori*

Virulence factors are important for understanding the relationship between the bacterial phenotype with specific manifestations of disease and elucidating mechanisms of pathogenesis. Virulent strains are accepted to be more aggressive and increase the risk of developing *H. pylori* related gastric diseases. Virulence factors with potential predictive value for specific pathologies include; VacA, CagA, OipA, HpaA, DupA, NapA, BabA, UreA, and UreB.

1.6.2.1 Vacuolating cytotoxin gene A (*vacA*)

Vacuolating cytotoxin A (VacA) is one of the most extensively studied toxins produced by *H. pylori*. Approximately 50% of *H. pylori* strains secrete this highly immunogenic 95-kDa protein. In addition to the induction of vacuolation, as named with reference to

form “vacuole-like” membrane vesicles in the cytoplasm of gastric cells, *vacA* has variety of effects on target cells, including colonization of *H. pylori*, cytochrome c release from mitochondria leading to apoptosis, disruption of mitochondrial functions, and binding to cell membrane which is followed by the initiation of a proinflammatory response, blockade of T-cell proliferation. Membrane channel formation, disruption of endosomal and lysosomal activity, effects of integrin receptor-induced cell signaling, interference with cytoskeleton-dependent cell functions, induction of apoptosis, and immune modulation are other activities of the VacA protein (Cover & Blanke, 2005).

The vacuolating cytotoxin gene A (*vacA*) is possessed by approximately 50 % of *H. pylori* strains, but the sequence heterogeneity within the *vacA* gene causes vacuolating activities among strains. There are two divergent regions: The signal region (s) and the middle region (m). Signal region has been grouped into three different types; s1a, s1b and s2 (Figure 1.4). The middle region, which contains the p58 cell binding domain, has been grouped into m1 and m2 (van Doorn *et al.*, 1998). Vacuolating activity is high in s1/m1 genotypes, intermediate in s1/m2 genotypes, and absent in s2/m2 genotypes (Atherton *et al.*, 1995). The intermediate region (i) region between the s and m region, has been identified in 2007. All s1/m1 strains were classified as i1 type, s2/m2 strains were classified as i2 type (Rhead *et al.*, 2007). Recently, d region, between the i and m regions has been identified. The d region is divided into d1 without deletion and d2 with a 69 to 81 bp deletion (Ogiwara *et al.*, 2009).

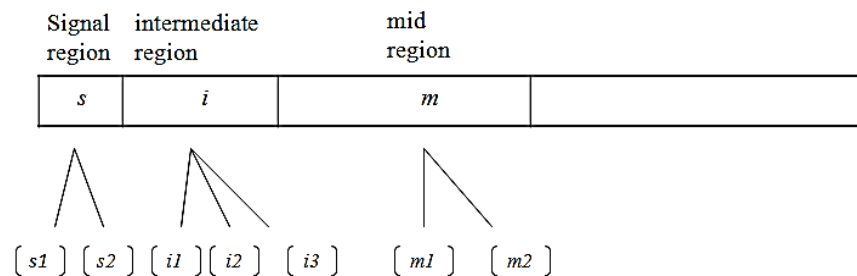


Figure 1.4 : Mosaic structure of the *vacA* gene; Three variable regions in the *vacA* gene sequence are the signal region (s region), mid- region (m region) and intermediate region (i region). There are two types of allelic variations in the s region and m region as s1, s2 and m1, m2. i1, i2 and i3 are the allelic variations in the intermediate region.

1.6.2.2 Cytotoxin- associated gene A (*cagA*)

Pathogenicity islands (PAIs) are horizontally acquired genetic elements that contain virulence genes. The cytotoxin associated gene (*cag*) PAI of *H. pylori* encodes a type IV secretory system that allows the translocation of effector molecules, such as CagA virulence factor, into the host cell (Covacci & Rappuoli, 2000). The *cag*-PAI is 40 kb in size and encodes approximately 30 genes which are multicistronic and involved in the biogenesis of type IV secretion system (Censini *et al.*, 1996). Moreover, expression of the genes within the *cag* PAI determines the differences in the ability of *H. pylori* strains to trigger chemokines from gastric mucosa (Atherton, 2000). Following *H. pylori* adherence to host cells, CagA is injected from bacterium into the bacterium-attached gastric epithelial cells and subsequent tyrosine phosphorylation by Src family kinases within epithelial cell where CagA functions as a scaffolding adaptor and interacts with at least ten host proteins that regulate cell growth, motility, polarity and intracellular signal transduction (Hatakeyama & Higashi, 2005).

CagA virulence factor is translocated into gastric epithelial cells. After translocation it localizes to the inner surface of the plasma membrane, in which it undergoes tyrosine phosphorylation at the Glu-Pro-Ile-Tyr-Ala (EPIYA) motif. EPIYA motifs found on the CagA are phosphorylated and induce strong immune response. The number of EPIYA motifs present on CagA varies amongst the strains. Strains with high motif number show more biological activity, more virulence and play a potential role in development of gastric cancer (Higashi *et al.*, 2002).

1.6.2.3 Cag type IV secretion system (Cag-T4SS)

Many gram-negative bacteria possess type IV secretion system that serves as a syringe-like pilus structure (also called T4SS pilus) protruding from the bacterial surface and is driven by host cell contact to acquire genetic materials, DNA-protein complexes or to inject virulence factors (Kwok *et al.*, 2007; Rohde *et al.*, 2003).

Type IV secretion system (T4SS) in *H. pylori* is responsible for injection of cytotoxin associated antigen A (CagA) across the bacterial cell envelopes into human gastric epithelial cells leading to pathogenesis (Kwok *et al.*, 2007). The delivery of CagA by the T4SS requires a host cell receptor, the integrin member β_1 and phosphatidylserine.

Numerous structural T4SS components have been demonstrated to bind to integrin β_1 including CagA, CagI and CagY but excluding CagL (Jimenez-Soto *et al.*, 2009; Murata-Kamiya *et al.*, 2010). CagL is responsible for the formation of the pilus tips of the T4SS that includes an arginine-glycine-aspartate (RGD) motif leading to bacteria to bind $\alpha_5\beta_1$ integrin on the surface of target cell. CagI is another protein which plays role in part of *cagPAI*. CagM protein is important for the translocation of CagA from the cytoplasm to the bacterial membrane. Non-Cag-PAI proteins have also been involved in the CagA translocation. HopQ is one of the non-Cag-PAI proteins which is required for the CagA translocation (Kumar *et al.*, 2013; Kwok *et al.*, 2007).

1.6.2.4 Outer inflammatory protein A (OipA)

The outer membrane proteins of *H. pylori* play an important role in pathogenesis. The outer inflammatory protein A (OipA) is one of those membrane proteins which plays the key role in the bacterial adhesion to gastric cell and development of inflammation. OipA functional status is regulated by slipped-strand mispairing within CT dinucleotide repeats in the 5' terminus of the gene. Variation in the number of CT dinucleotide repeats in the 5' terminus means that an active protein may or may not be produced. This slipped-strand repair mechanism based on the adaptation system is called phase variation. When there is 6, 9, (1 + 3), (2 + 3), (1 + 2), (1 + 1 + 1), (1 + 1 + 2) or other CT dinucleotide repeats, peptide kept in frame which means *oipA* gene is “on” status. Otherwise, the status is “off”, which leads to nonfunctional *oipA* gene. *OipA* “on” status is associated with more severe gastric diseases as ulcer and gastric cancer because of increased bacterial adherence and colonization (Kudo *et al.*, 2004; Yamaoka *et al.*, 2006).

1.6.2.5 Blood group antigen-binding adhesion protein A (BabA)

BabA is one of the major outer membrane proteins (OMPs) that mediates the binding of bacterium to the fucosylated Lewis b blood group antigen, Le^b, and related to terminal fucose residues found on blood group ABO antigens that are expressed on the surface of the gastric epithelial cell. Lewis b-dependent attachment of *H. pylori* to gastric epithelial cell is associated with increased severity of inflammation (Rad *et al.*, 2002). BabA

binding to Le^b is also important for induction of DNA double-strand breaks and DNA damage response in host cells (Toller *et al.*, 2011).

1.6.2.6 Duodenal ulcer promoting gene A (*dupA*)

The duodenal ulcer promoting gene A of *H. pylori* has been identified as a novel virulent marker associated with an increased risk for duodenal ulcer. The *dupA* is located in the plasticity region of the *H. pylori* that is homologous to *virB4* gene encoding a component protein of the T4SS in *A. tumefaciens*. DupA pathogenesis induces the IL-8 production from the antrum part of the stomach leading to antrum-predominant gastritis which is a characteristic of duodenal ulcer. Moreover, the presence of *dupA* was associated with increased mucosal neutrophil infiltration and its presence was inversely related to mucosal atrophy and gastric cancer (Lu *et al.*, 2005).

1.6.2.7 Neutrophil - activating protein A (NapA)

One of the most common finding in *H. pylori* infection is the infiltration of neutrophils and mononuclear cells within the gastric mucosa. There is a correlation between the neutrophil infiltration and mucosal damage (Davies *et al.*, 1992; Fiocca *et al.*, 1992). Neutrophil-activating protein A (NapA) is a water-soluble 150 kDa dodecameric protein that has been identified as a major *H. pylori* infection related virulence factor and promotes neutrophil infiltration to the site of the infection (Zanotti *et al.*, 2002). NapA promotes endothelial adhesion of neutrophils and stimulates high production of oxygen radicals from polymorphonuclear leukocytes (PMNs). Moreover *napA* increases the synthesis of tissue factor and secretion of type-2 plasminogen activator inhibitor by monocytes/macrophages. NapA is localized in the cytosol of bacteria and released upon autolysis. Following autolysis, it binds to the external surface of the outer membrane of bacteria to mediate the binding of *H. pylori* to the cell surface of host cell via interaction with carbohydrates (Cover *et al.*, 1994; Namavar *et al.*, 1998). The production of reactive oxygen radicals by induced neutrophils is a key component of the innate immune system. Also, neutrophils are an effective antimicrobial agent against *H. pylori* as well as a factor that maintains mucosal damage and gastritis (Evans *et al.*, 1995; Satin *et al.*, 2000).

1.6.2.8 Putative neuraminylactose-binding hemagglutinin homologue A (HpaA)

H. pylori adheres to the receptor on the surface of the gastric epithelial cells by its adhesin molecules. HpaA is a putative neuraminylactose-binding hemagglutinin protein A that enables the bacteria to adhere host gastric epithelial cells surface. HpaA has also been described as a flagellar sheated protein (Evans *et al.*, 1988; Evans *et al.*, 1993; Jones *et al.*, 1997). This protein acts as hemagglutinin and aggregates with assembly of fibrillar structure.

1.6.2.9 UreaseA gene (*ureA*) and UreaseB gene (*ureB*)

Urease is a pivotal enzyme for decreasing stomach acidity by generating ammonia and carbonate from urea (Hu & Mobley, 1990). This enzyme is necessary for *H. pylori* colonization on the gastric mucosa and a potent immunogen that draws out a vigorous immune response. *H. pylori* urease encoding genes are located as a single 6.13-kb gene cluster on the chromosome of the bacterium (Clayton *et al.*, 1990; Cussac *et al.*, 1992; Labigne *et al.*, 1991). For the synthesis of an active enzyme seven contiguous genes, *ureABIEFGH*, all transcribed in the same direction are necessary (Cussac *et al.*, 1992; Hu *et al.*, 1992; Hu & Mobley, 1993). *ureA* and *ureB* are the first two genes of the gene cluster and their expression is sufficient to produce an assembled apoenzyme. The active site of the enzyme is found in the UreB subunit. The UreA subunit of *Helicobacter* species is different from the other bacterial species because its amino acid sequence is encoded by the single *ureA* gene. Two separate genes are needed for the other species like *Escherichia coli* (Hu *et al.*, 1992).

1.7 Assays for Diagnosis of *H. pylori*

Diagnostic tests for *H. pylori* infection can be divided into two groups: invasive techniques, which require upper gastrointestinal endoscopy (gastroscopy) and are based on the analysis of gastric biopsy specimens, and non-invasive techniques such as serology, urea breath test, urine/blood test, or detection of *H. pylori* antigen in stool specimen.

1.7.1 Invasive tests

1.7.1.1 Endoscopic diagnosis

Endoscopic diagnosis is based on examination that requires the patient to be sedated before an endoscope equipped with miniature video equipment is inserted through the mouth and down into the upper part of the gastrointestinal tract, which includes the esophagus, stomach and duodenum. The gastroenterologist can then take a biopsy (sample of tissue) for pathological testing to determine the presence of *H. pylori* infection. Confocal endomicroscopy is one of the most impressive advances in the use of endoscopic techniques for visualization of *H. pylori* colonizing the gastric mucosa (Kiesslich *et al.*, 2005).

In the recent studies that describe gastric mucosal surface pattern with high magnification, a good correlation was reported between the histopathological findings with a sensitivity and specificity (91–100%) for *H. pylori* infection and gastric atrophy (Anagnostopoulos *et al.*, 2007; S. Kim *et al.*, 2006). However, the present position for endoscopy persists for the investigation of obstacle due to *H. pylori* and sampling biopsies for culture, histology, and rapid urease test.

1.7.1.2 Histology

Histologic testing is one of the most useful methods for detection of *H. pylori* infection. This test is considered as a gold standard. However, issues include size, number and site of the gastric biopsies, staining methods and experience of the examining pathologist make this test an imperfect gold standard for the detection of *H. pylori* infection (el-Zimaity, 2000). On the other hand, when histology is compared to other diagnostic tests it has advantages of evaluation for pathologic changes related with *H. pylori* infection such as inflammation, intestinal metaplasia, atrophy and malignancy (Dixon *et al.*, 1996).

1.7.1.3 Culture

Culture is used as a specific and standard diagnostic test for the most of the bacterial infections. However, it is time consuming and not necessary for the detection of relatively fastidious and slow growing microaerophilic *H. pylori*. Also, the sensitivity of

culture has been found to vary between the laboratories because of the *H. pylori* distribution in stomach and challenge in collecting biopsy sample. Other invasive tests like histology or rapid urease test are cheaper than the culture method (Makristathis *et al.*, 2004; Perez-Perez, 2000).

In addition to being a specific and standard diagnostic test, culture has advantage of being able to detect bacterial drug sensitivity. It has great value to decide the subsequent antimicrobial treatment if a patient does not give response to standard antimicrobial therapy (Lehours *et al.*, 2003).

1.7.2 Molecular methods

In the recent years, molecular tests have been widely used to detect *H. pylori* infection or *H. pylori* drug resistance based on the specific sequence of *H. pylori* DNA using different molecular techniques.

1.7.2.1 Polymerase chain reaction (PCR)

Many assays based on polymerase chain reaction (PCR) techniques have been employed to detect *H. pylori* DNA by using different gene targets from a variety of sources. PCR test has sensitivity and specificity over 95% as compared to the other tests (Lage *et al.*, 1995). However its application and optimization makes these tests more expensive. Detection of *H. pylori* by PCR is possible for the samples such as gastric biopsy, gastric juice, dental plaque, saliva, stool etc. Especially, virulence factors are the most important targets for PCR.

1.7.2.2 Quantitative real-time PCR

The detection of *H. pylori* DNA by real-time polymerase chain reaction (RT-PCR) is promising and there have been numerous studies to increase the sensitivity and specificity of this method. Especially to test the drug resistance, RT-PCR is preferred because of its quick and accurate results (Chey & Wong, 2007).

1.7.2.3 Multiplex-PCR

Multiplex-PCR is a technique that is used to amplify more than one target in a single PCR assay. For the rapid detection of *H. pylori* infection as well as virulence genes and

drug resistance from the gastric samples, multiplex-PCR test is used. In multiplex PCR, by using less DNA, two or more loci can be simultaneously amplified in the same reaction (Chattopadhyay *et al.*, 2004).

1.7.2.3.1 Optimization of Multiplex-PCR

Multiplex-PCR is a technique in which more than one target sequence is amplified using more than one pair of primers. The amount of reagents and preparation time is less in multiplex-PCR than uniplex-PCR in which several tubes are used. A multiplex-PCR method is ideal for conserving costly polymerase and templates in short supply (www.premierbiosoft.com). Even though it has many advantages, multiplex-PCR technique requires extensive optimization because of having many different parameters for each target. The most important steps in optimization of multiplex-PCR design are shown in Figure 1.5. The first step is optimizing all PCR parameters for single-target amplifications. Then, primers for each target are combined to determine which primer sets are compatible with each other. Also thermocycling parameters are optimized to get the best multiplex PCR products. The presence of more than one primer pair in the multiplex PCR increases the risk of obtaining non-specific amplification products, primarily because of the formation of primer dimers. Sometimes these nonspecific products are amplified more efficiently than the target product with consuming reaction components and producing impaired rates of annealing and extension. Primer design for multiplex PCR is a challenging problem. Several factors need to be considered. Primer pairs used in a multiplex PCR should enable similar amplification efficiencies for their respective target (Shen *et al.*, 2010). Thermocycling parameters and ingredients in PCR are other two factors that have to be optimized firstly for each target and then for each multiplex PCR combinations. Annealing temperature and PCR cycle number have to be adjusted according to the primers' T_m , primer and the product length. Ingredients in the multiplex PCR have an important effect on the PCR product.

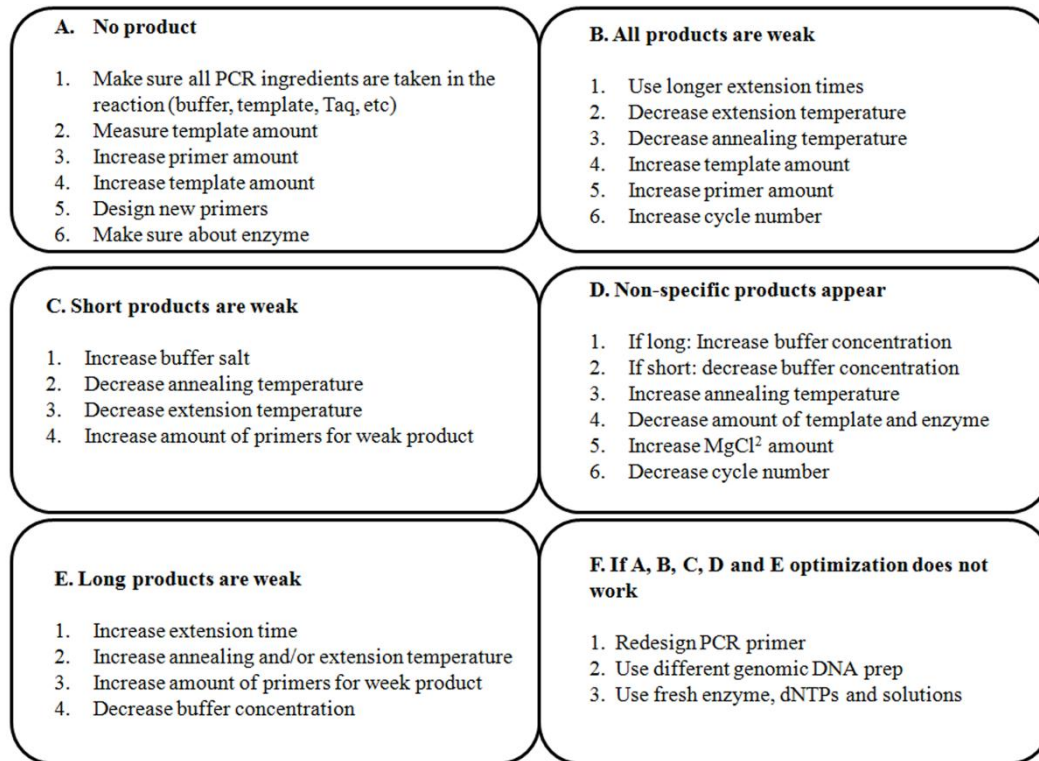


Figure 1.5 : Guidelines of multiplex-PCR optimization; Primer design, primer validation, primer concentration, optimization of amounts of dNTPs, MgCl₂, polymerase, salts and the thermalcycler parameters are the factors that have effect on the multiplex-PCR optimization. This guide can be used to troubleshoot the difficulties during the optimization of multiplex-PCR assays.

1.7.2.4 Stool PCR

H. pylori-infected individuals excrete *H. pylori* in feces; therefore the pathogen could be detected in stool specimens by PCR. However, false-negative and false-positive results are larger problem in the stool PCR, because stool samples include many chemicals, commensal bacteria and inhibitors. In contrast to the limitations, it is preferred compared to taking biopsy by the patients (Mapstone *et al.*, 1993; Namavar *et al.*, 1995).

1.7.2.5 Fluorescence in situ hybridization (FISH)

H. pylori specific DNA probes are used to detect bacterial infection in fluorescence in situ hybridization method. When FISH is compared to the other diagnostic methods, it is advantageous as it is not affected by DNA contamination and it allows direct

visualization of bacteria in the gastric biopsy specimens (Almeida *et al.*, 2009; Perry-O'Keefe *et al.*, 2001).

1.7.2.6 Rapid urease test (RUT)

RUT is a widely used biopsy-based method, in a clinical practice, to detect *H. pylori* urease enzyme which promotes the survival of bacteria in the acidic stomach environment by breaking down the urea into carbon dioxide and ammonia, in clinical practice (Chey & Wong, 2007). RUT detects the active *H. pylori* infection in a gastric biopsy sample using an agar gel or a reaction strip containing urea. It is a simple and a rapid test but its sensitivity can be decreased in patients with acute ulcer bleeding and usage of bismuth containing compounds and antibiotics (Lee *et al.*, 2000).

1.7.3 Non-invasive tests

A wide variety of non-invasive tests can be used for the initial diagnosis of *H. pylori* infection. Urine, whole blood, saliva, serum, expired air are used as a sample for these non-invasive diagnostic test.

1.7.3.1 Urea breath test (UBT)

Urea breath test is based on the analysis of enzyme activities and metabolic processes. The UBT has become available for clinical routine by the introduction of stable isotopes (Non radioactive ^{13}C labeled substances).

Normally, urea of noninfected individuals leaves stomach unchanged unless other bacteria's urease activities are present in the oral cavity. In *H. pylori* infected individuals, urea is degraded into ammonia and carbon dioxide. To detect the hydrolysis products of urea, it is labelled by different stable isotopes such as ^{13}C for $^{13}\text{CO}_2$ in the expired air and ^{15}N for detection of ^{15}N labelled ammonia in urine. To get the best specificity and sensitivity from urea breath test gastric pH, age, dose and application tracer, and interfering drugs should be considered (Graham *et al.*, 1987; Krumbiegel *et al.*, 2000). ^{14}C is also used for some breath tests, but its usage is not preferred for children because of its radiation (Koletzko *et al.*, 1998).

1.7.3.2 Stool antigen test (SAT)

Diagnosis of *H. pylori* infection in stool by using *H. pylori* antigen is another attractive non-invasive method which is very suitable for both clinical use and epidemiological research studies. The most common *H. pylori* antigen detection method in stool is enzyme immunoassay (EIA). Many new methods have been developed to detect *H. pylori* infection based on the stool test such as; polyclonal antibody EIA, monoclonal antibody EIA and immunochromatographic quick test.

The stool antigen tests are easy to perform, independent of age and cheaper compared with the UBT. This test method has advantage over several others of being easily applicable to children (Oderda *et al.*, 2000; van Doorn *et al.*, 2001; Yee *et al.*, 2002).

1.7.3.3 Antibody tests

H. pylori infection leads both local and systemic antibody responses in the body. After infection, a systemic response begins with the rising of specific IgM level and followed by IgA and IgG level increase throughout the infection. Detection of these antibodies are performed by enzyme linked immunosorbent assay (ELISA) or latex agglutination tests. Serum and urine are used as samples in these tests. The accuracy of antibody based diagnostic test is equal to the UBT (Alemohammad *et al.*, 1993; Weston *et al.*, 1995).

1.8 Treatment of *H. pylori* Infection

After the discovery of *H. pylori* and understanding its role in gastric diseases, management of patients with *H. pylori* has changed. *H. pylori* eradication needs multidrug therapy, typically antibiotics with an acid suppressant. Many drug therapies have been implemented to cure *H. pylori* infection. Unfortunately, drug resistance is largely responsible for treatment failures for the *H. pylori* infection. New drugs are needed to cope with increasing prevalence of antibiotic resistance among *H. pylori* (Hunt, 1997).

1.8.1 Standard first-line therapy

Standard first-line therapy includes two antibiotic; amoxicillin and clarithromycin to kill the bacteria and a proton-pump inhibitor (PPI) to enhance the antibiotic activity, which

is the most commonly appointed *H. pylori* eradication regimen (Sasaki *et al.*, 2013). However, this triple therapy may fail due to several reasons. The main reason for this therapy failure is resistance of *H. pylori* to clarithromycin (Megraud & Lamouliatte, 2003). The bacteriostatic activity of this antibiotic depends on its inhibition activity of the protein synthesis by binding to the 50S bacterial ribosomal subunit. The bacterial resistance to macrolides such as clarithromycin depends on the point mutations in the peptidyltransferase region encoded in domain V of 23S rRNA. These mutations lead to inhibition on the binding between the clarithromycin and the ribosomal subunit dedicated to the specific antibiotic related protein synthesis. Mutations associated with clarithromycin resistance are transition of adenine to cytosine in 2143 and 2142 positions of rRNA and the substitution of adenine with cytosine in 2142 positions of rRNA (Megraud, 2004; van Doorn *et al.*, 1999; Versalovic *et al.*, 1996).

1.8.2 Second and third-line therapies

After the first-line therapy failure an alternative second-line and third-line therapies can be used for the treatment of *H. pylori* infection. The second-line therapy includes proton-pump inhibitor (PPI), bismuth, tetracycline and metronidazole. Tetracycline is one of the protein synthesis inhibitor antibiotics that affect cell growth by inhibiting translation (Brodersen *et al.*, 2000). Metronidazole inhibits nucleic acid synthesis by disrupting DNA of microbial cells. When *H. pylori* loses oxygen-insensitive NADPH nitroreductase activity which reduces an active metabolite directly toxic to the bacteria, resistance against to metronidazole is developed (Tomb *et al.*, 1997). Levofloxacin-based triple therapy consisting of levofloxacin, amoxicillin and a PPI represents an encouraging strategy for second-line therapy (Saad *et al.*, 2006). The triple therapy includes PPI, amoxicillin and sitafloxacin. Amoxicillin inhibits synthesis of bacterial cell wall (Geddes *et al.*, 2007). Sitafloxacin is a fluoroquinolone that inhibits the topoisomerase II in the bacterial cell, leads to DNA fragmentation via nuclease activity of the intact enzyme domains (Andersson & MacGowan, 2003).

1.8.3 Sequential and concomitant (Non-bismuth Quadruple) therapy

The most widely recommended treatment for *H. pylori* infection is triple therapy consists of a proton pump inhibitor-based (PPI) with two antibiotics (Chey & Wong,

2007; Coelho *et al.*, 2000; Gisbert *et al.*, 2005; Lam & Talley, 1998; Malfertheiner *et al.*, 2007). However, eradication rate of the triple therapy has considerably fallen (Graham *et al.*, 2007). Because of the antibiotic resistance, scientists have been trying to develop new alternative treatment regimens against the *H. pylori* infection.

Sequential treatment includes 5 days of PPI treatment plus amoxicillin, followed by a further 5 days of PPI together with two other antibiotics, usually clarithromycin and metronidazole (Gisbert *et al.*, 2010). Due to recent research, sequential regimen is more effective than standard triple therapy (Gatta *et al.*, 2009; Jafri *et al.*, 2008; Moayyedi, 2007; Tong *et al.*, 2009; Zullo *et al.*, 2007).

Concomitant (Non-Bismuth quadruple) treatment includes three clarithromycin, amoxicillin and metronidazole or tinidazole together with the PPI for the duration of therapy. Concomitant treatment is an effective, safe, and well-tolerated alternative to triple therapy and also this treatment is less complex than sequential therapy (Graham & Shiotani, 2008).

1.8.4 Levofloxacin and other fluoroquinolones

Levofloxacin is one of the fluoroquinolones that shows its function on type II topoisomerase enzymes. One of the type II topoisomerase enzymes affected by levofloxacin is DNA gyrase which is responsible for supercoiling the DNA. When there is an effect on this enzyme; DNA cannot be supercoiled and does not fit the newly duplicated cells. The other enzyme is a topoisomerase IV which is necessary to separate DNA that has been replicated prior to bacterial cell division. When replicated DNA is not separated, the bacterium cannot divide (Drlica & Zhao, 1997; Mutschler *et al.*, 2001). Another and newer fluoroquinolone agent used for *H. pylori* therapy is sitafloxacin. It has *in vitro* activity against gram-negative bacteria including *H. pylori* (Sanchez *et al.*, 2000).

1.8.5 Bismuth-based therapies

Bismuth is a heavy metal that is used as anti-*H. pylori* agent due to its effect on bacterial iron uptake. Bismuth is used as combination with metronidazole and tetracycline or amoxicillin in the triple therapy (de Boer, 1999) and it was reported that this therapy

cures 87.9% of patients within one week of treatment and 89.2% of patients within 2 weeks of treatment (Tytgat *et al.*, 1990).

1.8.6 Probiotics

Antimicrobial treatments for *H. pylori* infection are highly effective, but it is expensive. Antibiotics have side effects and sometimes antibiotic resistance can be a considerable problem. Probiotics are used as an alternative solution to treat *H. pylori* infection. Probiotic bacteria can act on *H. pylori* by the help of several putative mechanisms; intake of probiotics strengthens the nonimmunological barrier of stomach; produces antimicrobial substances to inhibit *H. pylori* colonization, stimulates mucin production, and stabilizes the gut mucosal barrier (Jack *et al.*, 1995; Vandenberg, 1993). Also, attachment of *H. pylori* to gastric epithelial surface can be inhibited by probiotics. Probiotics can lead to increase in the amount of mucin secretion of stomach and modifications in the immunologic response of the host (Gill, 2003). *L. acidophilus* 4356, *L. casei* 193, *L. lactis* BH5, *B. subtilis*, *W. confusa* PL9001 are some probiotics microorganisms used for the treatment of *H. pylori* infection (Rolfe, 2000).

1.9 Immune Response to *H. pylori* Infection

The host immune response against bacterial pathogen is divided into innate and adaptive response. When the host is infected with a bacterial pathogen innate immune response shows a quick and non-specific process and reacts with bacterial molecules to signal infectious danger aiming to kill bacteria (Portal-Celhay & Perez-Perez, 2006). Toll-like receptors (TLRs) expressed on antigen-presenting cells (APCs) such as dendritic cells (DCs) and monocytes mediate innate immune response towards bacterial infections. When bacteria contact with APCs and monocytes proinflammatory cytokines are secreted such as tumor necrosis factor α (TNF- α), Interleukin 8 (IL-8) and Interleukin 1 β (IL-1 β). For *H. pylori* infection, these cytokines act as local chemo-attractants and induce granulocytic infiltration (Crabtree, 1996). When failure of innate immune response about elimination of pathogen occurs, adaptive immune response elicits a delayed, antigen specific response and activates T-, B- and memory cells.

The adaptive immune response can be divided into cellular and humoral immune response. Inflammatory reactions caused by *H. pylori* are defined by a mucosal infiltration of many cells like polymorphonuclear leukocytes (PMN), T cells, plasma cells, and macrophages. Adhesion of *H. pylori* to gastric mucosa and secretion of different molecules change gastric epithelial cell function (Ihan *et al.*, 2012). As an humoral response, *H. pylori* infection induces specific systemic and local antibody response. Also, infected individuals secrete antibodies against the bacteria (Mattsson *et al.*, 1998).

1.9.1 T cells' development and types of T cells

T cells are a subset of lymphocytes that play a central role in cell-mediated immunity. They can be distinguished from other lymphocytes, such as B cells and natural killer cells (NK cells), as having a T-cell receptor (TCR) on their cell surface. T-cell receptors detect fragments of antigens on the surfaces of infected or cancerous cells (Alberts, 2002). T cell development and maturation involve a complex variety of interactions with non-lymphoid cell products and receptors. All progenitors of T cells originate from haematopoietic stem cells in the bone marrow. Haematopoietic progenitors enter the thymus and differentiate, undergo selection, and eventually mature into functional T cells (Schwarz & Bhandoola, 2006). Complex transcriptional network, specific receptor-ligand pair interactions and sensitization to trophic factors play role in this process that mediates homing, proliferation, survival and differentiation of developing T cells (Koch & Radtke, 2011). In the thymus, T cells develop their specific T cell markers, including TCR, CD3, CD4 or CD8, and CD2. CD4⁺ T cells are classified into two main classes; T helper cell 1 (Th1) and T helper cell 2 (Th2) on the basis of cytokines and transcription factors. T helper 17 (Th17), T helper 9 (Th9) and T helper 22 (Th22) are newly described CD4⁺ T cells that have a crucial role in the pathogenesis of various immune diseases (Figure 1.6).

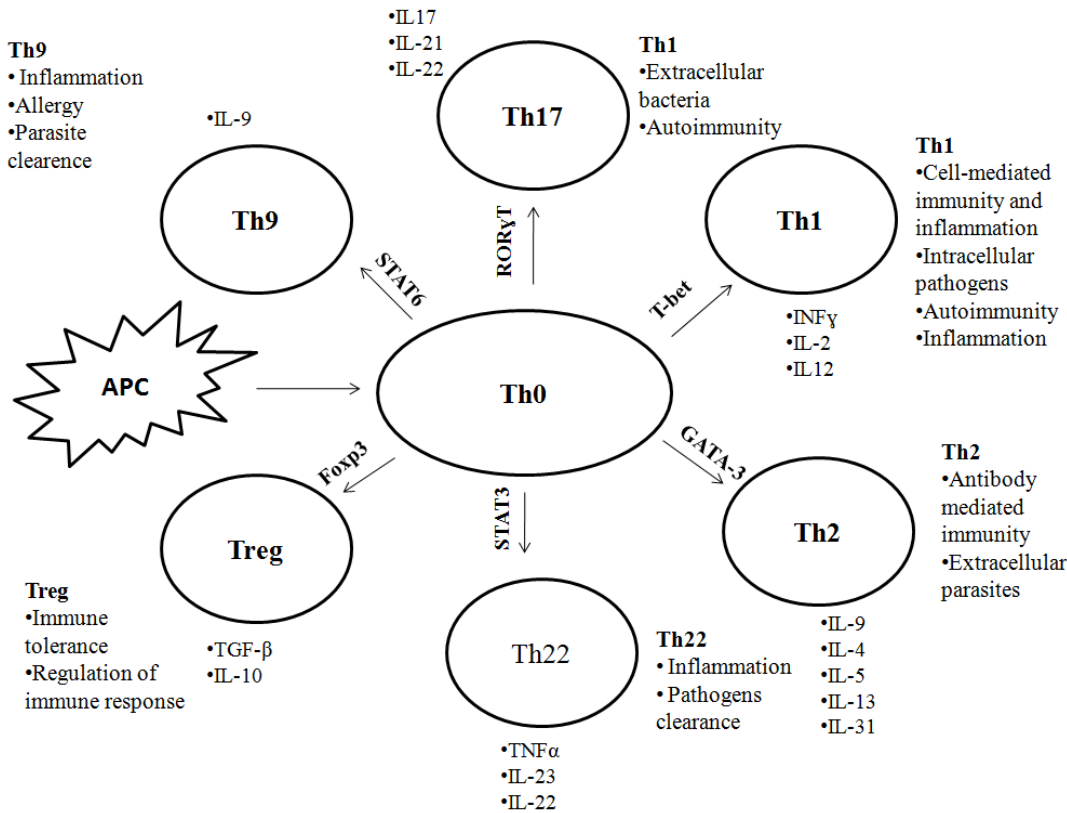


Figure 1.6 : CD4⁺ T helper cells differentiation; functions, unique products, characteristic transcription factors and cytokines critical for fate determination.

1.9.1.1 T helper 1 cell (Th1)

One of the first described T helper cells are T helper 1 cells (Th1). Th1 cells are a lineage of CD4⁺ effector T cells that secrete signature cytokine interferon-γ (IFN-γ), Interleukin 2 (IL-2) along with other pro-inflammatory cytokines, like tumour necrosis factor-α (TNF-α) and TNF-β. Th1 cells induce macrophage activation, nitric oxide production, and cytotoxic T lymphocyte proliferation, leading to phagocytosis and destruction of microbial pathogens. Interleukin 27 (IL-27), interleukin 12 (IL-12) and IFN-γ are cytokines that take part in Th1 cell differentiation and expansion together with a subset of specific cell surface receptors including IL-12 R beta 2, IL-27 R alpha/WSX-1, IFN-γ R2, CCR5 and CXCR3. STAT1-dependent expression of T-bet, Th1-specific transcription factor and a master regulator of Th1 differentiation are induced by IL-27 signaling in naive CD4⁺ T cells. T-bet is a member of T-box family of transcription factors and is the only known T-box gene that specifically expressed in the lymphoid

system. T-bet assists expression of IFN- γ and IL-12 R beta 2. IL-12 R beta 2 heterodimerizes with IL-12 R beta 1 to make a functional IL-12 receptor complex that then stimulates STAT4-dependent IFN- γ production and Th1 differentiation (J. Zhu & Paul, 2008).

IFN- γ has been accepted as an important cytokine that play role in the differentiation of Th1 cells. However, Interleukin-12 (IL-12) is also a potent inducer of Th1 differentiation (Hsieh *et al.*, 1993; Seder *et al.*, 1993; Swihart *et al.*, 1995; Wang *et al.*, 1994; Wenner *et al.*, 1996). Both IFN- γ and IL-12 function through promoting expression of Th1-specific transcription factors that are STAT1, STAT4, and T-bet.

Th1 cells promote cell-mediated immunity characterized by cellular cytolytic activity and protect the host from the obligate intracellular pathogens. On the other hand, excessive pro-inflammatory activities of Th1 can lead tissue damage and elicit unwanted inflammatory diseases as well as autoimmune disorders such as insulin-dependent diabetes mellitus and rheumatoid arthritis disease and graft-versus-host disease (Davidson *et al.*, 1996; Hu *et al.*, 1999; Leung *et al.*, 2000; B. Wang *et al.*, 1997).

IFN- γ , also known as Type II interferon or immune interferon, is a cytokine that is critical for innate and adaptive immunity against viral, bacterial and protozoal infections. This cytokine promotes protection against intracellular pathogens. IFN- γ is produced primarily by CD4⁺ T helper 1 (Th1) and CD8⁺ cytotoxic T lymphocyte (CTL) as part of antigen-specific immunity and by natural killer (NK) and natural killer T (NKT) cells once innate immune response develops (Schoenborn & Wilson, 2007).

IFN- γ activates macrophages' effector functions, affects isotype switching and potentiates the secretion of immunoglobulins by B cells. Also, this cytokine upregulates expression of class I and II major histocompatibility complex (MHC) molecules, Fc receptor and leukocyte adhesion molecules those are important for adaptive immune response against pathogens. IFN- γ promotes differentiation of naive T helper cells into Th1 cells and suppresses Th2 cell differentiation. IFN- γ acts by binding to specific cell surface receptors with high-affinity binding sites (Schroder *et al.*, 2004).

1.9.1.2 T helper 2 cell (Th2)

T helper type 2 cells are a distinct lineage of CD4⁺ effector T cell that secretes IL-4, IL-5, IL-9, IL-13, IL-10 and IL-17E/IL-25. Th2 cells are associated with humoral immunity and important in coordinating the immune response to large extracellular pathogens. IL-4 is the most potent cytokine that is required for Th2 priming, maturation and differentiation (Le Gros *et al.*, 1990; Swain *et al.*, 1990). GATA binding protein 3 (GATA-3) is a member of the GATA family of transcription factors that is required for Th2 differentiation (Ouyang *et al.*, 1998; Pai *et al.*, 2004). The high concentrations of Th2 can block generation of Th1 cells from naive T cells.

1.9.1.3 T helper 17 Cell (Th17)

T helper 17 cell is a newly identified, independent subset of T helper cells. Interleukin 17 (IL-17) cytokine family, including IL-17A (commonly referred to as IL-17), IL-17B, IL-17C, IL-17D, IL-17E (also known as IL-25) and IL-17F are secreted by Th17 cells. Th17 cells play an important role in host defense against fungal and bacterial infection, especially at mucosal surfaces. IL-17 and IL-22 production triggers generation of Th17 cell, which improves mucosal barrier surfaces by stimulating release of antimicrobial peptides and recruiting neutrophils (Harrington *et al.*, 2005). Both transforming growth factor- β (TGF- β) and IL-6 act cooperatively and non-redundantly to improve Th17 commitment. IL-6 induces Th17 cells along with TGF- β (Zhou *et al.*, 2007). Retinoic acid-related orphan receptor gamma (ROR γ) is a “master-regulator” transcription factor that controls differentiation of Th17 cells. ROR- α and ROR- γ t are critical in promoting Th17 cell differentiation (Jin *et al.*, 2010).

IL-17 is a potent proinflammatory cytokine that intensifies ongoing inflammation by activating expression of tumor necrosis factor- α (TNF- α), IL-1 β , and IL-6 in epithelial and endothelial cells. IL-17 is the signature cytokine of T helper 17 cells. IL-17 is produced by natural killer cells after stimulation of T-cell antigen receptor (Weaver *et al.*, 2007).

Human IL-17 gene has 1874 base pairs in length and it was derived from CD4⁺ T cells (Yao *et al.*, 1995). There are several proteins homologous to IL-17, which includes IL-17B, IL-17C, IL-17D, IL-17E, and IL-17F. Expressions of IL-17A and IL-17F appear to

be restricted to a small group of activated T cells and upregulated during inflammation. Expression of IL-17B occurs in several peripheral tissues and immune tissues. IL-17C is upregulated in inflammatory conditions and IL-17D is highly expressed in the nervous system and in skeletal muscle. IL-17E is expressed at low levels in various peripheral tissues (Aggarwal & Gurney, 2002).

RAR-related Orphan Receptor Gamma t (ROR γ t) is one of the members of the nuclear receptor family of intracellular transcription factors that play critical roles during homeostasis and specific stages of development. ROR- α , - β , and - γ are three forms of RORs and they have different roles. ROR- α plays role in the development of the cerebellum and lymph nodes, lipid metabolism, immune response, and maintenance of bone. ROR- β is highly expressed in the brain and retina, but the exact role is not known. ROR- γ is participated in lymph node development and immune response and survival of T helper 17 cells. Differentiation of Th17 requires transforming growth factor-beta but also depends on distinct transcription factors such as ROR γ t (Hirose *et al.*, 1994). ROR γ t is selectively expressed in Th17 cells differentiated in the presence of TGF- β plus IL-6 and transduction of naive T cells with retroviral vector containing ROR γ t induces the development of Th17 cells (Ivanov *et al.*, 2006).

1.9.1.4 T helper 9 cell (Th9)

T helper 9 cells that are characterized by expression of high level of interleukin 9 (IL-9) have been recently described. Th9 cells enhance the pro-inflammatory Th17 cell- driven immune response and prevent allograft rejection by intermediating the T cell tolerance (Annunziato & Romagnani, 2009).

1.9.1.5 T helper 22 cell (Th22)

T helper 22 (Th22) cells are subset of human CD4⁺ effector T cells that primarily secrete IL-22, IL-13, and TNF-alpha. Th22 cells have been found in the epidermis of inflammatory skin disorders and they are believed to be important to contribute to the host defense against microbial pathogens and promote tissue repair or remodeling (Basu *et al.*, 2012).

1.9.2 Regulatory T cell (Treg)

Regulatory T (Treg) cells, known as suppressor T cells, form a subset of CD4⁺ T cells that develop in the thymus (nTreg; naturally occurring) or comprehend from naive T cells in the presence of TGF- β ensuing T-cell receptor stimulation (iTreg; induced Treg) in the periphery. Treg cells are immune-suppressive cells that are distinct from other immune response promoting Th cells. The most important function of Treg cells is maintaining self-tolerance and immune homeostasis (Sakaguchi, 2004; Shevach, 2000). Also, Treg cells are crucial for tempering immune responses against infectious agents and renewing immune homeostasis after pathogen clearance (Belkaid, 2008). Forkhead Box P3 (FOXP3) is a transcription factor expressed in Treg cells. FOXP3 is known as the master regulator of Treg function. Tregs control expression of a wide array of genes including cytokines and surface molecules playing a suppressive role in the immune system (Zheng & Rudensky, 2007).

FOXP3 acts as master switch gene in the development and function of regulatory T cells (Zhang & Zhao, 2007). FOXP3 transcription factor occupies the promoter region of genes that involved in regulatory T-cell function and could suppress transcription of key genes subsequent to stimulation of T cell receptors (Marson *et al.*, 2007). Molecular mechanisms behind the expression of FOXP3 gene are incompletely understood. However, it is known that TGF- β , IL-2 or TCR stimulation of T cells can all result in increased FOXP3 expression (Kim & Leonard, 2007).

1.9.3 The role of T cells in the *H. pylori* infection

CD4⁺ T helper cells can be differentiated to Th1, Th2, Th17 and Treg cells according to the induction of various cytokines and transcription factors. Especially, Th1 and Th17 cells together with Tregs play important roles on the *H. pylori*-induced immune response. The number of Th1, Th2 and Th17 cells increase in *H. pylori*-infected gastric lamina propria. These cells play important roles in pathogenesis of bacterial infection by increasing inflammation (Razavi *et al.*, 2015).

In general, Th1 cell responses are induced most by intracellular bacteria, whereas Th2 cell responses are stimulated by extracellular parasites including helminths. Previously, it was shown that gastric lymphocyte populations from *H. pylori*-infected patients

contain increased IFN- γ -producing T cells, consistent with a Th1 cytokine response. Moreover, *H. pylori*-specific T cell clones derived from gastric mucosa of the patients with peptic ulcer disease had a Th1-profile (Ekkens *et al.*, 2007). Abundant levels of Th1 cytokines, IFN- γ and IL-2, and low levels of Th2 cytokines, IL-4 and IL-5, are produced by the T cells harvested from *H. pylori*-infected individuals. The adaptive immune response to *H. pylori* is composed of both Th1 and Th2 type cells, but cytokine profiles indicate predominance of a Th1 response in *H. pylori*-infected patients. The Th1 proliferation in *H. pylori*-infected gastric mucosa involves signals provided by antigen-presenting cells and cytokines produced in response to the components of the pathogen, such as lipopolysaccharide, resulting in increased secretion of IFN- γ , IL-12, and IL-18 (Larussa *et al.*, 2015).

A novel subset of effector T cells, pro-inflammatory Th17 cells, is identified by secretion of IL-17A, IL-17F, IL-21, and IL-22. By promoting chronic inflammation, Th17 cells are clearly implicated in the pathogenesis of autoimmune diseases and in the protective immunity against extracellular bacterial infections. However, the protective and pathogenic functions of IL-17 producing Th cells were also reported in infections. Several publications have reported that, *H. pylori* infection increases IL-17 in the gastric mucosa. IL-17 production has shown to be crucial for neutrophil recruitment to the *H. pylori*-infected gastric mucosa (Shi *et al.*, 2010).

H. pylori-specific regulatory T cells (Treg) suppress elevated activation of effector CD4⁺ T cells as well as effector CD8⁺ T cells. This leads to control of gastric inflammation and persistent colonization of the bacterium. As reported in a recent study, *H. pylori*-infected individuals have increased number of regulatory T cell (Treg) cells in the stomach and duodenal mucosa compared to uninfected controls (Lundgren *et al.*, 2013). Treg cells are present in the mucosa of individuals with asymptomatic *H. pylori* infections as well as in duodenal ulcer patients. Also, the frequency of Treg cells increase in the stomachs of *H. pylori*-infected patients with gastric adenocarcinoma (Lundgren *et al.*, 2005). These findings suggest that Treg cells could suppress mucosal immune responses and contribute to the persistence of *H. pylori* infections (Larussa *et al.*, 2015).

1.10 Hypothesis

H. pylori has been recognized as the main risk factor for gastritis, peptic ulcer, gastric carcinoma and gastric mucosa-associated lymphoma. The clinical outcomes of *H. pylori* infection is highly variable and is influenced by bacterial virulence factors, host genetic factors, and immune responses. *H. pylori* virulence factors have been correlated with specific manifestations of the disease and expound mechanisms of pathogenesis. The important *H. pylori* virulence factors, which play roles in the disease process, are the cytotoxin associated gene A (*cagA*), vacuolating cytotoxin A (VacA), outer inflammatory protein A (OipA), blood group antigen-binding adhesion (BabA), putative neuraminylactose-binding hemagglutinin homolog A (HpaA), neutrophil activating protein A (NapA), duodenal ulcer promoting gene A (*dupA*), urease subunit A (UreA), urease subunit B (UreB). Another factor that is important in *H. pylori*-driven disease process is the host's immune response. Both CD4⁺ T effector cells and CD4⁺CD25⁺ regulatory T (Tregs) cells are increased in gastric mucosa and peripheral blood of *H. pylori* infected patients. The impact of virulence factors on the interplay between CD4⁺ T cells and *H. pylori* have not been elucidated in detail. Therefore, the aim of the study was to investigate the influence of nine major *H. pylori* specific virulence factors; CagA, VacA, OipA, BabA, HpaA, NapA, DupA, UreA, UreB on CD4⁺ T cell response (Th1, Th17 and Treg) in *H. pylori*-infected gastritis and ulcer patients. Firstly a multiplex-PCR assay for the simultaneous detection of nine selected *H. pylori* virulence factors was developed. Secondly, the expression levels of Th1, Th17, and Treg specific cytokines and transcription factors were assessed in *H. pylori* infected gastritis and ulcer patients. Finally, expert-derived models for diagnostic prediction of gastric diseases using *H. pylori* virulence factors and host immune responses, was developed.

2. MATERIALS AND METHODS

2.1 Materials and Laboratory Equipments

2.1.1 Equipment

The laboratory equipments used in this study were listed in Appendix A.

2.1.2 Chemicals and kits

The chemicals and kits used in this study were given in Appendix B together with their suppliers. The preparation and compositions of buffers and solutions were shown in Appendix C.

2.2 Selection of Patients

Biopsy specimens were obtained from the patients who underwent endoscopy due to gastroduodenal -complaints at the Gastroenterology Department of Acibadem Hospital Groups, in Istanbul, Turkey. In total, 109 (87 with gastritis and 22 with ulcer) patients were selected who fulfilled the following criteria for obtaining expulsion: under 18 years, 65 years or older patients with active infection, cancer patients, patients with inflammatory diseases, patients who have had gastrointestinal bleeding within the last month and who have had previous gastrointestinal surgery, patients diagnosed with chronic liver failure, known renal failure, diabetic patients, pregnant women and patients who have previously received *H. pylori* eradication treatment, immunosuppressive therapy (including steroids), patients who have had nonsteroidal anti-inflammatory drugs (NSAIDs) and / or antibiotic treatment in the last three weeks, antisecretory therapy in the last two weeks and patients who refused to sign the informed consent form. However, 29 of 109 patient samples were then eliminated after they were found to be *H. pylori* negative with urease PCR and histopathological staining; thus, 80 patients (62 with gastritis and 18 with ulcer) were included in this study. Each participant

provided a written informed consent to participate to the study and the ethical committee of Acıbadem University and Istanbul Technical University approved the study. Eighteen participants in the control group were selected from the patients who underwent routine endoscopic examination and had negative rapid urease test results for *H. pylori*.

2.3 Gastric Biopsy Specimens

Two gastric biopsy specimens were taken from the antrum and corpus parts of the stomach of the patients during endoscopy, one for DNA isolation and the other for RNA isolation. Fresh biopsy specimens were placed into a preservative solution (Ambion, RNAlater® RNA Stabilization Solution), kept overnight at + 4°C, and then placed at – 80°C until DNA and RNA isolation.

2.4 RNA Extraction

To study gene expression, RNA was isolated from RNALater-stabilized human tissue specimens. Frozen biopsy specimens were homogenized by using a high efficiency homogenization system (SpeedMill PLUS, Analytikjena) and total RNA was isolated using RNA isolation kit, following the protocol recommended by the manufacturer (innuSPEED tissue RNA kit Analytikjena). All RNA samples were quantified using NanoDrop (ND-2000, ROCHE) and maintained in – 80 °C deep freezer until cDNA synthesis. cDNA was synthesized using 1 µg of RNA through a reverse transcription reaction (High Capacity cDNA Reverse Transcription 127 Kit, Applied Biosystems).

2.5 DNA Extraction

To study virulence factors of *H. pylori*, DNA was extracted immediately after RNA isolation by using a DNA isolation kit (Quick g-DNA™ 130, ZYMO RESEARCH) following the manufacturer's description. All DNA samples were quantified using NanoDrop (ND-2000, ROCHE) and maintained in – 80 °C deep freezer. Also, integrity of the DNA samples was assessed with the gel electrophoresis.

2.6 Primer Design

Primers, obtained from Metabion International AG, Germany were used in this study. For the PCR assay primers, GenBank entries were searched for the selected virulence genes sequences including *ureA*, *ureB*, *cagA*, *oipA*, *hpaA*, *babA*, *dupA*, and *napA*. The primers were designed by using Primer 3 software (Table 2.1). For amplification of *vacA s1/s2*, *vacA m1/m2* genes previously published PCR primers (Table 2.1) were used. For the quantitative RT-PCR assay primers, GenBank entries were searched for sequences of the genes encoding human IFN- γ , T-bet, IL-17, ROR γ t, FOXP3. The published sequences were aligned and primers were designed by using the LightCycler probe design software (Roche Diagnostics, Mannheim, Germany) (Table 2.2). A BLAST search was performed to confirm the specificity of the DNA sequences of all the primers ("The National Center for Biotechnology Information,").

Table 2.1 : Multiplex-PCR primers designed for the amplification of *H. pylori* virulence genes.

DNA region(s)	Primer Name	Sequences (5'→3) PCR amplified	Product Size (bp)	References
<i>ure A</i>	ure A-F	TGA TGG GAC CAA CTC GTA ACC GT	244	This study
	ure A-R	CGC AAT GTC TAA GCG TTT GCC GAA		
<i>ure B</i>	Ure B-F	AGT AGC CCG GTG AAC ACA ACA TCCT	645	This study
	Ure B-R	ATG CCT TTG TCA TAA GCC GCT TGG		
<i>cag A</i>	Cag A-F	AGAGCAAGCGTTAGCCGATCTCAA	415	This study
	Cag A-R	TTTCCCTACACCACCCAAACCACT		
<i>hpa A</i>	hpa A-F	TAG TGG GAT GCA GCC CGC ATA TTA	534	This study
	hpa A-R	CGC TAT GGC TTG AAT GGG TGG TTT		
<i>oip A</i>	Oip A-F	GTT TTT GAT GCA TGG GAT TT	401	(Veralovic, 1991)
	Oip A-R	GTG CAT CTC TTA TGG CTT T		
<i>bab A</i>	Bab A-F	AAT CCA AAA AGG AGA AAA AGT ATG AAA	832/601	(Pavithran, 2002)
	Bab A-R	TGT TAG TGA TTT CGG TGT AGG ACA		
<i>dup A</i>	dupA-F	TGA GCG TGG TAG CTC TTG AC	584	This study
	dup A R	GAG CGC GTT AGC GAT ATA GG		
<i>nap A</i>	napA-F	GAA TGT GAA AGG CAC CGA TT	304	This study
	napA-R	ATC GTC CGC ATA AGT TAC GG		
<i>vac A s1/s2</i>	vac A s1/s2-F	ATG GAA ATA CAA CAA ACA CAC	259/286	(Atherton <i>et al.</i> , 1995)
	vac A s1/s2-R	CTG CTT GAA TGC GCC AAA C		
<i>vac A m1/m2</i>	vac A m1/m2-F	CAA TCT GTC CAA TCA AGC GAG	567/642	(Atherton <i>et al.</i> , 1999)
	vac A m1/m2-R	GCG TCTVAAA TAA TTC CAA GG		

Table 2.2 : Quantitative RT-PCR primers designed for the detection of Th1,Th17 and Treg cell response.

Primer Name	Sequences (5'→3)	References
IL-17-F	CCTGGGAAGACCTCATTGGT	This study
IL-17-R	ATTCCAAGGTGAGGTGGATCG	
ROR γ t-F	CTGCAAAGAAGACCCACACC	This study
ROR γ t-R	GCAGTTCTGCTGACGGGT	
IFN- γ -F	TCCAAAAGAGTGTGGAGACCA	This study
IFN- γ -R	TCGACCTCGAAACAGCATCT	
FOXP3-F	TGACAGTTTCCCAACAAGCCA	This study
FOXP3-R	GAAGATCTCGGCCCTGGAAG	

2.7 Optimization of Multiplex-PCR Assay

To optimize the concentrations of the conventional multiplex-PCR components, the specificity of each primer pair and the thermocycling parameters for each virulence factor were tested individually in separate reactions by using genomic DNA isolated from *H. pylori* G27 strain as a positive control. Thermal cycling parameters, such as annealing time and temperature, extension time and temperature, cycling number were optimized for each primer pair. Also, the PCR assay mixture parameters were optimized. Obtaining the best amplification depends on template, buffer and dNTPs and Mg⁺⁺ concentration. Mg⁺⁺ concentration optimization was done for each primer. Also, to enhance the fidelity of polymerization without reducing the yield of PCR, different amounts of dNTPs were studied. Also different polymerase enzymes and PCR master mixes were studied to get the best PCR amplification. After all these optimizations, different primer pair combinations were studied to find the most compatible for multiplex-PCR. Each thermal cycling parameters and PCR assay mixture parameters were optimized for each primer pair.

2.8 Multiplex-PCR Assay

Genomic DNA isolated from *H. pylori* G27 strain used in this study was a kind gift from Prof. Dr. Anne Mueller from University of Zurich, Institute of Molecular Cancer Research, Switzerland. Genomic DNA of *H. pylori* G27 strain and DNA obtained from gastric specimens were used as template DNAs in multiplex-PCR assays. The amplification reactions were carried out in a total volume of 25 μ l and amounts used in reaction are given in Table 2.3. PCR conditions performed are given in Table 2.4. The

PCR products were subjected to electrophoresis on agarose gels and stained with SYBR Gold (Invitrogene). The specificity of the primer pairs was confirmed by employing *H. pylori* G27 strain as a positive control.

Table 2.3 : PCR assay components.

Component	Amount
10X DreamTaq Buffer (includes 20 mM MgCl ₂),	2,5 µl
dNTP (200 µM)	2,0 µl
Frw Primer (20 µM)	1 µl
Rev Primer (20 µM)	1 µl
Dream Taq DNA polymerase (0,65 U)	0,69 µl
PCR Grade water	18,81 µl

Table 2.4 : Reaction conditions of multiplex-PCR.

Temperature	Time
95°C	3 min.
95°C	45 sec.
60 °C	45 sec. (45 cycles)
72°C	2 min.
72°C	5 min.
4°C	∞

2.9 Quantitative Real-Time PCR

Optimal annealing temperatures of the qRT- PCR primer pairs and expected product size were determined with conventional RT-PCR. SYBR Green qRT-PCR amplifications were performed using a LightCycler 480 Real-Time Detection System (ROCHE). All qRT-PCR experiments were carried out in duplicate with a reaction volume of 10 µl, using 96-well optical grade PCR plates (ROCHE) covered with optical-quality sealing film (ROCHE). All qRT-PCR experiments were carried out three times independently. Negative controls without cDNA and positive controls for these genes were included in the experiments. The efficiency of qRT-PCR amplification was optimized for each primer pair, using various dilution series of cDNA. Amounts used in reaction are given in Table 2.5. PCR conditions performed are given in Table 2.6. The percentage of expression of the cytokines and the transcription factors were calculated from quantitative RT-PCR results based on the cycle threshold (Ct). Relative quantification method was used to analyze the PCR. Relative quantification describes the change in expression of the target gene relative to the Hp negative control group. $2^{-\Delta\Delta CT}$ Method

is used to calculate relative changes in the gene expression determined by the quantitative RT-PCR and as an internal control 18S rRNA housekeeping gene primers were used to normalize the quantitative RT-PCR. 95% confidence intervals (CIs) were calculated to measure the positivity of the IFN- γ (Th1), IL-17 (Th17), and FOXP3 (Treg) on samples from patients with gastritis and ulcer. 95% confidence intervals (CIs) were calculated as the average of the sample plus two-standard deviation (95% CI= average + 2 σ) for the samples in the control group that were *H. pylori* negative. Samples over the 95% confidence intervals were accepted as positive. And correlations of these results with the virulence factors were adapted to expert derived models.

Table 2.5 : PCR assay components.

Component	Amount
Power Sybr Master Mix (2X)	5 μ l
Frw Primer (10 μ M)	0.5 μ l
Rev Primer (10 μ M)	0.5 μ l
PCR Grade water	1.5 μ l

Table 2.6 : Reaction conditions of Real-Time PCR.

Temperature	Time
95°C	10 min.
95°C	15 sec.
Depends on primer	1 min. (40 cycles)
72°C	1,5 min.
72°C	5 min.
4°C	∞

2.10 Multiplex Urease PCR Assay

For rapid identification of *H. pylori* with urease (ure) gene-based PCR assay, genomic DNA from several biopsy samples and primer sequences were evaluated. *ureA* and *ureB* are the target genes for the urease activity of *H. pylori*. To optimize the multiplex urease PCR genomic DNA isolated from *H. pylori* G27 strain is used. The amplification reactions were carried out in a total volume of 25 μ l and amounts used in reaction are given in Table 2.7. PCR conditions performed are given in Table 2.8. The PCR products were subjected to electrophoresis on agarose gels and stained with SYBR Gold (Invitrogene). The specificity of the urease primer pairs was confirmed by employing a positive and several negative controls.

Table 2.7 : PCR assay components.

Component	Amount
10X DreamTaq Buffer (includes 20 mM MgCl ₂),	2,5 µl
dNTP (200 µM)	2,0 µl
Frw Primer (20 µM)	1 µl
Rev Primer (20 µM)	1 µl
Dream Taq DNA polymerase (0,65 U)	0,69 µl
PCR Grade water	18,81 µl

Table 2.8 : Reaction conditions of multiplex-urease PCR.

Temperature	Time
95°C	3 min.
95°C	45 sec.
60 °C	45 sec. (45 cycles)
72°C	2 min.
72°C	5 min.
4°C	∞

2.11 Statistical Analysis

Statistical analysis of correlation between *H. pylori* virulence factors and gastric disease type (i.e. Gastritis or Ulcer) was performed using two-tailed Fisher's exact test to examine the significance of the association. Additionally, correspondence between virulence factor presence and immune response was assessed with Pearson product-moment correlation coefficients. However, observing a strong correlation by chance is not uncommon for small sample sizes. Therefore, interpretation of correlation coefficients without any significance testing may be misleading. Statistical significance of sample correlation coefficients (r) was assessed using two-tailed t-test under null model $\rho = 0$, where ρ denotes population correlation coefficient. Test statistic for this test was calculated as following:

$$t = \frac{r}{\sqrt{\frac{1-r^2}{N-2}}}$$

where N denotes sample size. Associated p-value is calculated using a t-distribution with $N-2$ degrees of freedom (Evans *et al.*, 1996). Statistical significance of correlations between virulence factors and immune response factors was assessed using a t-test.

To understand the performance of the predictive expert-derived models confusion matrices that reports the number of false positives, false negatives, true positives,

and true negatives were used. Differences at $P < 0.05$ were accepted as statistically significant. All calculations were performed using R (version 3.1.0, The R Foundation for Statistical Computing, Vienna, Austria; <http://www.r-project.org>) ("The R Foundation for Statistical Computing,").

2.12 Expert-Derived Models for Diagnostic Prediction

Classification of samples into gastritis and ulcer classes by using the virulence factors and the immune response results was performed by expert-derived model. The model is built on a randomly selected training data to discover potentially predictive relationships. The randomly selected training data consisted of two-thirds of the all dataset. Model building was performed by determining the most important feature first that can distinguish the ulcer and gastritis patients, and adding other features if they improve the overall accuracy of the prediction. Then best model was selected and validated on a test set. Remaining one third of the data was randomly selected as testing set, and this process is, repeated for 1000 times. Classification performance was assessed using prediction accuracy for each class. Mean of accuracies and their standard deviations were reported. All calculations were performed using R (version 3.1.0, The R Foundation for Statistical Computing, Vienna, Austria; <http://www.r-project.org>) ("The R Foundation for Statistical Computing"). Confusion matrix was calculated for the expert-derived models to show the number of correct and incorrect predictions ability of the models. Each column of the confusion matrix represents the instances in a predicted class while each row represents the instances in an actual class or vice-versa (Stehman, 1997).

2.13 Evaluation of The Functional Status of *oipA* Gene

The *oipA* was characterized by CT dinucleotide repeats in the signal-coding region of the gene. A PCR sequencing-based methodology was applied to detect the number of the CT dinucleotides repeats in the *oipA* genes and, therefore, to attempt assessment of its expression status. The PCR amplifications were performed in a total volume of 25 μ l and amounts used in reaction are given in Table 2.3. PCR conditions performed are given in Table 2.4. The resulting PCR products were subjected to gel electrophoresis in

a 2% agarose gels and stained with SYBR Gold (Invitrogene). Sequences of amplicons were directly determined, with the same forward and reverse primers employed as in the PCR assays, using the High-throughput Sanger sequencing (GATC BIOTECH).





3. RESULTS

3.1 Detection of *H. pylori* by Multiplex Urease PCR

Two subunit genes, *ureA* and *ureB* encode urease, which is the enzyme that contributes to the survival of bacteria in the acidic environment of the stomach. To determine the presence of *H. pylori* in human gastric biopsy specimens, we assessed the expression status of *ureA* and *ureB* by multiplex-PCR assay. We utilized *H. pylori* strain G27 with a known complete genome sequence (Tomb *et al.*, 1997) to optimize multiplex-PCR to detect *H. pylori* virulence factor genes, *ureA* and *ureB* genes. As a result of optimization studies, *ureA* and *ureB* genes were detected in a single PCR assay (Figure 3.1).

Next, we used multiplex-urease PCR assay to detect the presence of *H. pylori* in gastric biopsy specimens of 109 patients. In total 80 of these patients were detected as *H. pylori* positive.

Furthermore, multiplex-urease PCR results were compared with methods used in routine, the rapid urease test and histopathological staining. To analyse the efficiency, specificity and accuracy of multiplex-urease PCR assay compared with the rapid urease test and histopathological staining, Cohen's kappa coefficient value which measures inter-rater agreement for categorical items, was calculated for each comparison (Figure 3.2). If the raters are in complete agreement then $\kappa = 1$. The κ value can be interpreted as follows; $\kappa < 0$ "poor agreement", $\kappa = 0.00-0.20$ "slight agreement", $\kappa = 0.21-0.40$ "fair agreement", $\kappa = 0.41-0.60$ "moderate agreement", $\kappa = 0.61-0.80$ "substantial agreement", $\kappa = 0.81-1.00$ "almost perfect agreement" (Weston *et al.*, 1995). There was a substantial agreement between the results of multiplex-urease PCR and the histopathological staining. Cohen's kappa coefficient for the comparison of histopathological staining and rapid urease test results indicate a moderate agreement. There is a fair agreement between the multiplex urease PCR and the rapid urease test

results in which the κ is between 0.21-0.40 (Figure 3.2). Furthermore, the multiplex-urease PCR can detect *H. pylori* in some samples that are identified as *H. pylori* negative by the rapid urease test and histopathological staining method.

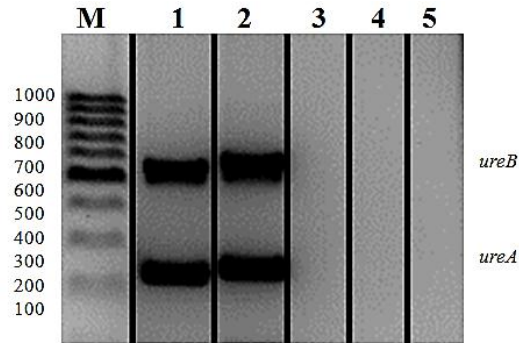


Figure 3.1 : Multiplex urease-PCR assay to detect the *H. pylori* positive and negative samples; Lane M, 100 bp ladder- marker (ThermoSCIENTIFIC, Gene Ruler), Lane 1 amplification of *ureA* and *ureB* genes of *H. pylori* positive control strain G27; between Lane 2 to 5 are from randomly selected patients. Lane 2 is *H. pylori* positive patient sample. Lane 3 to 5 are *H. pylori* negative samples.

Multiplex urease PCR vs Histopathological staining		
	Histopathological staining	
Multiplex urease PCR	Present	Absent
Present	69	13
Absent	0	27
$\kappa = 0.73$ ('substantial' agreement)		
Histopathological staining vs Rapid urease test		
	Rapid urease test	
Histopathological staining	Present	Absent
Present	54	15
Absent	6	34
$\kappa = 0.60$ ('moderate' agreement)		
Multiplex urease PCR vs Rapid urease test		
	Rapid urease test	
Multiplex urease PCR	Present	Absent
Present	54	28
Absent	6	21
$\kappa = 0.34$ ('fair' agreement)		

Figure 3.2 : Comparison of multiplex urease PCR test results with the rapid urease test and histopathological staining. For each comparison Cohen's kappa coefficient was used for calculation.

3.2 Optimization of Multiplex-PCR Conditions for *H. pylori* Virulence Factors

H. pylori-specific virulence factors influence the microorganism's pathogenicity. Many multiplex-PCR assays have been reported in the literature to detect the *H. pylori*-specific virulence factors that affect clinical outcomes of the infection (Shiota *et al.*, 2013). However, it is the first reported multiplex-PCR assay that can detect nine *H. pylori*-specific virulence factors in three PCR assay. In this study, it was aimed to simultaneously detect the genes that encode various virulence factors in *H. pylori*, namely babA, ureA, ureB, hpaA, cagA, oipA, dupA, napA and vacA, by multiplex-PCR. First, we attempt to detect seven *H. pylori*-specific virulence factors babA, ureA, ureB, hpaA, cagA, oipA and vacA by multiplex-PCR assay (Figure 3.3 A). To decrease the number of reactions while maximizing the number of detected virulence factors,

different primer combinations with different thermal cycling parameters were tested in a well-characterized *H. pylori* strain, G27. However, some of primers for certain virulence factors could not be amplified in the same PCR assay. For instance, oipA primers could not be amplified with the ureA, ureB and hpaA primers as shown in the first reaction of the Figure 3.3 B and different primer combinations were assessed to get best results for each virulence factors. While we attempt to optimize multiplex PCR assay, two *H. pylori* virulence factors, napA and dupA, were added to multiplex-PCR assay based on recently published data indicating their clinical importance. To find the most compatible primer combinations for the detection of virulence factors, different primer combinations with different thermalcycling parameters were tested (Figure 3.3 A and B). At the end of the optimization studies, nine virulence factors were detected with in three PCR assays; five in one, three in one and one in one (Figure 3.3 C). Unfortunately, babA virulence factor primers could not be combined with any of the other virulence factor primers.

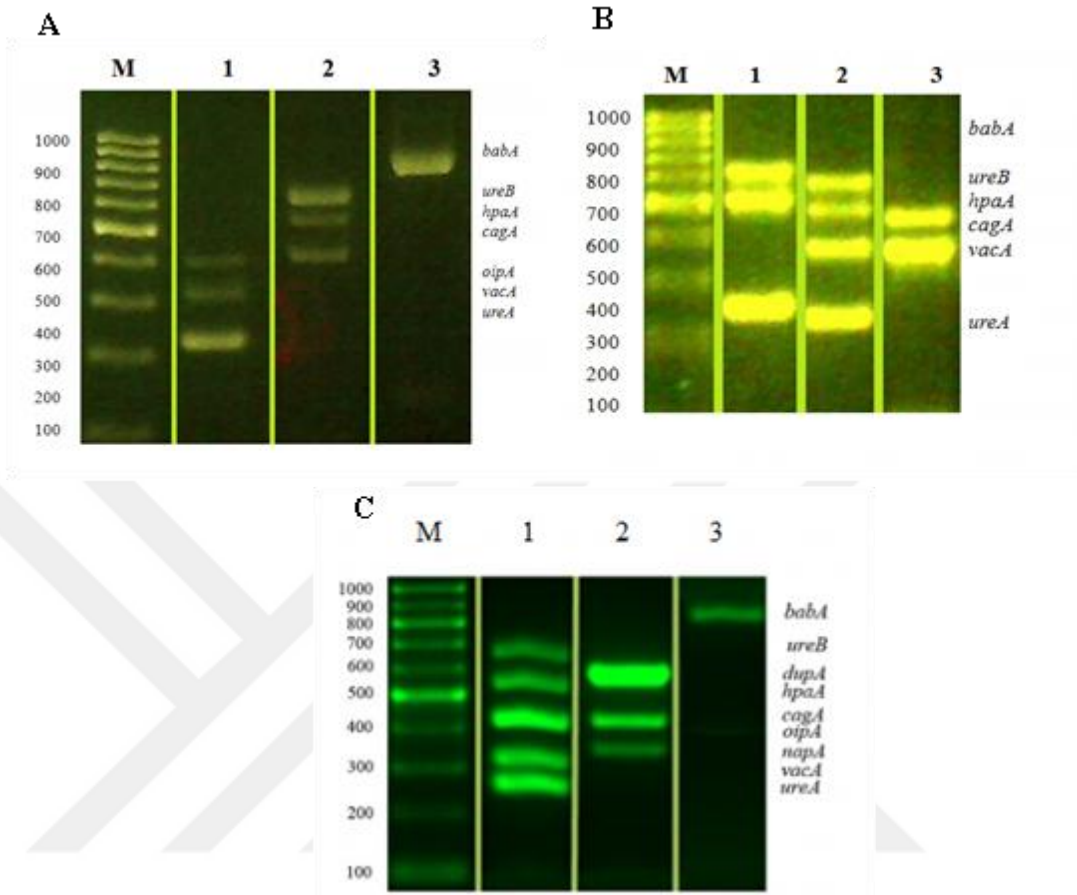


Figure 3.3 : Optimization of multiplex-PCR conditions. (A) Representative multiplex PCR data for amplification of different combinations of *H. pylori* virulence genes under different PCR conditions; Lane M, 100 bp-marker (ThermoSCIENTIFIC, Gene Ruler), Lane 1 (Reaction 1); oipA (401bp), vacA (333bp) ureA (244bp), Lane 2 (Reaction 2); ureB (645bp), hpaA (534bp), cagA (415bp) and Lane 3 (Reaction 3); babA(832bp). (B) Lane M, 100 bp- marker (ThermoSCIENTIFIC, Gene Ruler), Lane 1 (Reaction 1); ureB (645bp), hpaA (534bp), oipA (401bp), ureA (244bp), Lane 2 (Reaction 2) ureB (645bp), hpaA (534bp), cagA (415bp) , ureA (244bp), Lane 3 (Reaction 3); hpaA (534bp), vacA (333bp). (C) amplification of virulence genes of *H. pylori* G27 strain by multiplex PCR; Lane M, 100 bp- marker (ThermoSCIENTIFIC, Gene Ruler), Lane 1 (Reaction 1); ureA (244bp) , ureB (645bp), hpaA (534bp), cagA (415bp), napA (384bp), Lane 2 (Reaction 2); dupA (584bp), oipA (401bp), vacA (333bp), and Lane 3 (Reaction 3); babA(832bp).

3.3 Detection of *H. pylori* Virulence Factors by Multiplex-PCR

As a result of optimization studies, it was possible to detect nine *H. pylori* virulence genes within three PCR assays; *ureB*, *hpaA*, *cagA*, *napA*, *ureA* in a single reaction, *dupA*, *oipA*, *vacA* in a single reaction, and *babA* in a separate reaction (Figure 3.4 A) by using the genomic DNA derived from *H. pylori* G27 strain. Next, the multiplex-PCR was applied to genomic DNA derived from total of 80 *H. pylori* positive patients; 18 with ulcer and 62 with gastritis. The images shown in Figure 3.4 B are from a representative gel electrophoresis of a multiplex- PCR data of nine *H. pylori* virulence factors. In order to detect *H. pylori vacA* subtypes in *vacA* positive gastric specimens, four separate PCR assays were performed (Figure 3.3 C) and representative data for *vacA* subtypes in *vacA* positive patients with ulcer and gastritis are shown in Figure 3.4 D. Also virulence factors were tested on the negative control biopsy samples taken from *H. pylori* negative patients by multiplex-PCR and there was no PCR product for these samples (Figure 3.4 E).

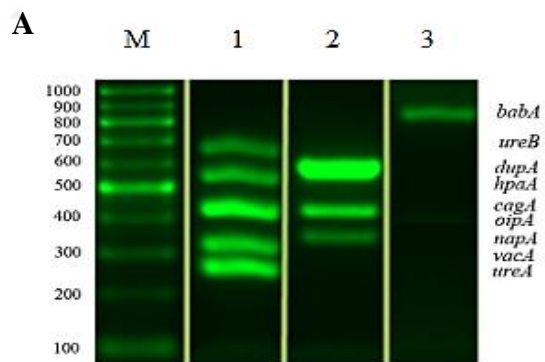


Figure 3.4 : Multiplex- PCR assay for *H. pylori*-specific virulence factors; (A) amplification of virulence genes of *H. pylori* G27 strain by multiplex PCR; Lane M, 100 bp- marker (ThermoSCIENTIFIC, Gene Ruler), Lane 1 (Reaction 1); *ureA* (244bp) , *ureB* (645bp), *hpaA* (534bp), *cagA* (415bp), *napA* (384bp), Lane 2 (Reaction 2); *dupA* (584bp), *oipA* (401bp), *vacA* (333bp), and Lane 3 (Reaction 3); *babA*(832bp).

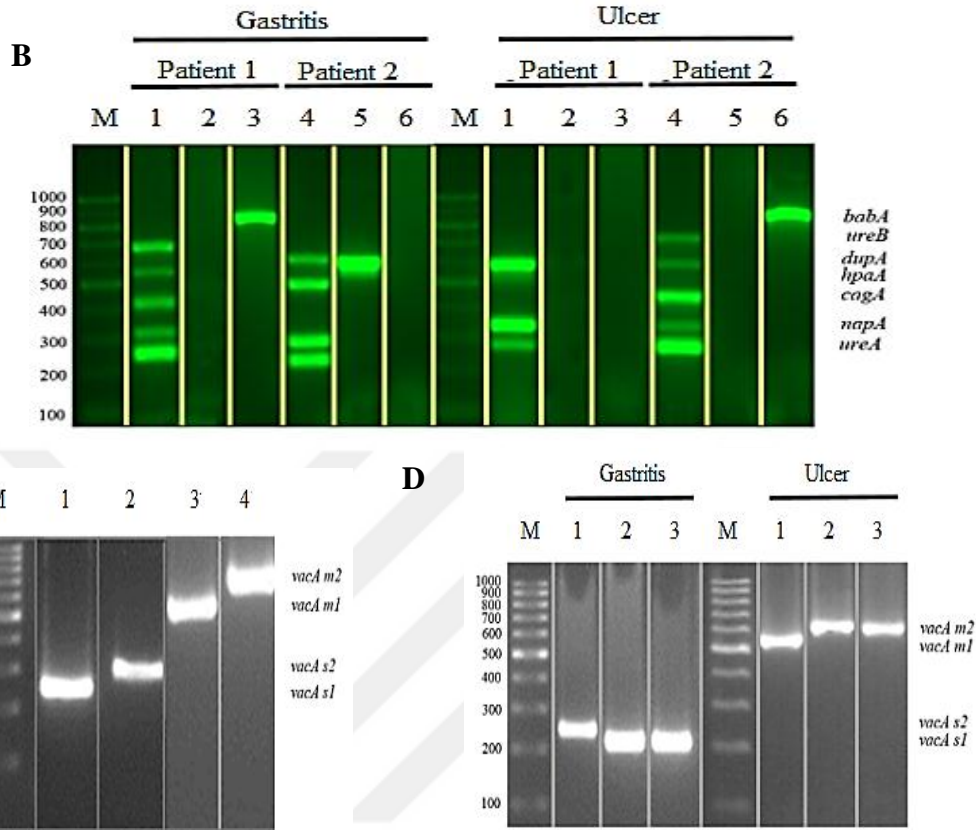


Figure 3.4 (continued) : Multiplex- PCR assay for *H. pylori*-specific virulence factors; (B) Multiplex-PCR application of the biopsy samples taken from randomly selected patients with ulcer and gastritis; between Lane 1 to 6 are representing patients with gastritis or ulcer: Multiplex PCR assay results of patient 1 and 2 (gastritis or ulcer) are shown between Lane 1 to 3 and Lane 4 to 6, respectively. Reaction 1 (Lane 1 and 4) ureA (244bp), ureB (645bp), hpaA (534bp), cagA (415bp), napA (384bp), Reaction 2 (Lane 2 and 5); dupA (584bp), oipA (401bp), vacA (333bp), and Reaction 3 (Lane 3 and 6); babA(832bp) Multiplex- PCR assay for *H. pylori*-specific virulence factors. (C) PCR inferred results of s1/s2 and m1/m2 alleles of vacA gene; Lane 1 (reaction 1); vacA s1 (259bp), Lane 2 (Reaction 2); vacA s2 (286 bp), Lane 3 (Reaction 3); vacA m1 (567 bp), and Lane 4 (Reaction 4) vacA m2(642 bp). (D) PCR inferred results of s1/s2 and m1/m2 alleles of vacA gene of randomly selected patients with gastritis and ulcer respectively; between Lane 1 to 3 are representing patients with gastritis (left side) or ulcer (right side): Patients with gastritis; Lane 1-3, vacA s1 (259bp), and vacA s2 (286 bp), patients with ulcer; Lane 1-3, vacA m1 (567 bp), and vacA m2 (642 bp).

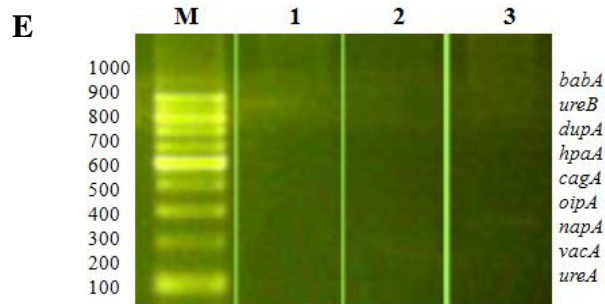


Figure 3.4 (continued) : Multiplex- PCR assay for *H. pylori*-specific virulence factors; (E) Multiplex-PCR application of the biopsy samples taken from *H. pylori* negative patients; Lane 1 (Reaction 1); *ureA* (244bp), *ureB* (645bp), *hpaA* (534bp), *cagA* (415bp), *napA* (384bp), Lane 2 (Reaction 2); *dupA* (584bp), *oipA* (401bp), *vacA* (333bp), and Lane 3 (Reaction 3); *babA*(832bp) Sybr Gold (Invitrogene) was used for the gels and gel pictures were taken by using Observable Real Time Gel Electrophoresis System (Salubris Technica, Turkey).

3.4 Correlation between the Virulence Factors in Patients with Gastritis and Ulcer

H. pylori-specific virulence factors have been found to be associated with increased risk of gastric diseases. So far, these virulence factors have been implicated in the inflammatory response towards *H. pylori* by different mechanisms like disturbing tissue homeostasis in epithelial cells, host-signaling pathways or stimulating innate immune cells (Wroblewski *et al.*, 2010). However, there is not enough information in the literature, about the association between the virulence factors in patients with gastritis and ulcer. To understand the relationship between various *H. pylori*-specific virulence factors, PCR was applied to genomic DNA derived from total of 80 *H. pylori* positive patients, 18 with ulcer and 62 with gastritis. Nine *H. pylori*-specific virulence factors; CagA, VacA, OipA, BabA, HpaA, NapA, DupA, UreA, UreB were studied to analyse the putative link between presence or absence of virulence factors with clinical outcomes of diseases associated with *H. pylori* infection as gastritis and ulcer. Association between the detected *H. pylori* virulence factors were measured by Pearson product-moment correlation coefficients that shows the linear relationship between two sets of data. The significance was determined by two-tailed test. To reduce the chances of obtaining false-positive correlation, Bonferroni correction was applied to Pearson product-moment correlation coefficients data. Based on the Pearson product-moment

correlation coefficients, there were many negative and positive correlations between virulence factors of *H. pylori* in patients with gastritis and ulcer. When the Bonferroni correlation correction was applied to the data, it was shown that there was a significant positive correlation between CagA-HpaA (r value = 0,47 and Bonferroni corrected p value = 7,01E-03) and NapA-VacA s2 (r value = 0,43 and Bonferroni corrected p value =4,61E-02) virulence factors and negative correlation between VacA m1-VacA m2 (r value = -0,82 and Bonferroni corrected p value = 2,87E-15) virulence factors in patients with gastritis. For the ulcer patients, there was a significant negative correlation between VacA m1-VacA m2 virulence factors (r value = -0,84 and Bonferroni corrected p value = 0,01) (Figure 3.5 A, B, C and D). All correlations are significant at the level $p \leq 0.05$.

A

Correlation (Pearson r) Gastritis													
	CagA	HpaA	OipA	BabA	VacA s1	VacAs2	VacA m1	VacA m2	ureA	ureB	dupA	napA	
CagA	1,00	0,47	0,33	0,19	0,16	-0,03	0,30	-0,23	-0,08	0,23	0,12	0,09	
HpaA	0,47	1,00	0,37	0,09	0,42	0,21	0,18	0,03	-0,16	0,37	0,18	0,21	
OipA	0,33	0,37	1,00	0,36	0,35	-0,08	0,25	-0,18	-0,21	0,33	0,14	-0,09	
BabA	0,19	0,09	0,36	1,00	0,05	0,02	0,22	-0,14	-0,11	-0,12	0,07	0,18	
VacA s1	0,16	0,42	0,35	0,05	1,00	-0,38	0,39	-0,27	-0,13	0,25	0,18	0,03	
VacA s2	-0,03	0,21	-0,08	0,02	-0,38	1,00	-0,18	0,22	0,10	0,11	-0,12	0,43	
VacA m1	0,30	0,18	0,25	0,22	0,39	-0,18	1,00	-0,82	-0,04	0,21	0,16	-0,05	
VacA m2	-0,23	0,03	-0,18	-0,14	-0,27	0,22	-0,82	1,00	0,15	-0,20	-0,10	0,13	
ureA	-0,08	-0,16	-0,21	-0,11	-0,13	0,10	-0,04	0,15	1,00	-0,17	0,09	0,15	
ureB	0,23	0,37	0,33	-0,12	0,25	0,11	0,21	-0,20	-0,17	1,00	0,19	-0,13	
dupA	0,12	0,18	0,14	0,07	0,18	-0,12	0,16	-0,10	0,09	0,19	1,00	0,27	
napA	0,09	0,21	-0,09	0,18	0,03	0,43	-0,05	0,13	0,15	-0,13	0,27	1,00	

Figure 3.5 : Pearson product-moment correlation coefficients and Bonferroni correction of *H. pylori* virulence factors correlation data. (A) Pearson product-moment correlation coefficients of virulence factors in gastritis. r values closer to +1 means there is positive correlation between data and r values closer to -1 means there is negative correlation between data in the Pearson product-moment correlation coefficients. Green colored values in the Bonferroni correction data show the significant positive correlation between data. Red colored values in the Bonferroni correction data show the significant negative correlation between data. NA means not applicable.

B

Correlation Significance (Two-tailed, P-value, Bonferroni Corrected)-Gastritis												
	CagA	HpaA	OipA	BabA	VacAs1	VacAs2	VacAm1	VacAm2	UreA	UreB	DupA	NapA
CagA	1,00	7,01E-03	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00
HpaA	7,01E-03	1,00	0,58	1,00	0,46	1,00	1,00	1,00	1,00	0,69	1,00	1,00
OipA	1,00	0,58	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00
BabA	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00
VacAs1	1,00	0,46	1,00	1,00	1,00	0,64	0,06	1,00	1,00	1,00	1,00	1,00
VacAs2	1,00	1,00	1,00	1,00	0,64	1,00	1,00	1,00	1,00	1,00	1,00	4,61E-02
VacAm1	1,00	1,00	1,00	1,00	0,06	1,00	1,00	2,87E-15	1,00	1,00	1,00	1,00
VacAm2	1,00	1,00	1,00	1,00	1,00	1,00	2,87E-15	1,00	1,00	1,00	1,00	1,00
UreA	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	0,00	1,00	1,00
UreB	1,00	0,69	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	0,00	1,00
DupA	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	0,00	1,00
NapA	1,00	1,00	1,00	1,00	1,00	4,61E-02	1,00	1,00	1,00	1,00	1,00	0,00

C

Correlation (Pearson r) Ulcer												
	CagA	HpaA	OipA	BabA	VacA s1	VacA s2	VacA m1	VacA m2	UreA	UreB	DupA	NapA
CagA	1,00	0,29	-0,02	0,32	0,04	-0,18	-0,02	-0,14	NA	0,28	NA	0,06
HpaA	0,29	1,00	0,13	0,01	0,25	0,18	-0,10	0,02	NA	0,15	NA	0,40
OipA	-0,02	0,13	1,00	-0,07	-0,12	0,22	-0,10	0,05	NA	0,02	NA	0,02
BabA	0,32	0,01	-0,07	1,00	-0,03	0,06	0,13	-0,03	NA	0,02	NA	0,08
VacA s1	0,04	0,25	-0,12	-0,03	1,00	-0,40	0,23	-0,22	NA	0,40	NA	0,14
VacA s2	-0,18	0,18	0,22	0,06	-0,40	1,00	-0,05	0,11	NA	-0,26	NA	0,44
VacA m1	-0,02	-0,10	-0,10	0,13	0,23	-0,05	1,00	-0,84	NA	0,02	NA	0,20
VacA m2	-0,14	0,02	0,05	-0,03	-0,22	0,11	-0,84	1,00	NA	-0,15	NA	-0,21
UreA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
UreB	0,28	0,15	0,02	0,02	0,40	-0,26	0,02	-0,15	NA	1,00	NA	0,27
DupA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
NapA	0,06	0,40	0,02	0,08	0,14	0,44	0,20	-0,21	NA	0,27	NA	1,00

Figure 3.5 (continued): Pearson product-moment correlation coefficients and Bonferroni correction of *H. pylori* virulence factors correlation data. (B) Bonferroni correction of gastritis data (C) Pearson product-moment correlation coefficients of virulence factors in ulcer. r values closer to +1 means there is positive correlation between data and r values closer to -1 means there is negative correlation between data in the Pearson product-moment correlation coefficients. Green colored values in the Bonferroni correction data show the significant correlation between data. Red colored values in the Bonferroni correction data show the significant negative correlation between data. NA means not applicable.

D

Correlation Significance (Two-tailed, P-value, Bonferroni Corrected)													
	CagA	HpaA	OipA	BabA	VacAs1	VacAs2	VacAm1	VacAm2	UreA	UreB	DupA	NapA	
CagA	0,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	NA	1,00	NA	1,00	
HpA	1,00	0,00	1,00	1,00	1,00	1,00	1,00	1,00	NA	1,00	NA	1,00	
OipA	1,00	1,00	0,00	1,00	1,00	1,00	1,00	1,00	NA	1,00	NA	1,00	
BabA	1,00	1,00	1,00	0,00	1,00	1,00	1,00	1,00	NA	1,00	NA	1,00	
VacAs1	1,00	1,00	1,00	1,00	0,00	1,00	1,00	1,00	NA	1,00	NA	1,00	
VacAs2	1,00	1,00	1,00	1,00	1,00	0,00	1,00	1,00	NA	1,00	NA	1,00	
VacAm1	1,00	1,00	1,00	1,00	1,00	1,00	0,00	0,01	NA	1,00	NA	1,00	
VacAm2	1,00	1,00	1,00	1,00	1,00	1,00	0,01	0,00	NA	1,00	NA	1,00	
UreA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	
UreB	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	NA	0,00	NA	1,00	
DupA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	
NapA	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	NA	1,00	NA	0,00	

Figure 3.5 (continued): Pearson product-moment correlation coefficients and Bonferroni correction of *H. pylori* virulence factors correlation data. (D) Bonferroni correction of ulcer data. r values closer to +1 means there is positive correlation between data and r values closer to -1 means there is negative correlation between data in the Pearson product-moment correlation coefficients. Green colored values in the Bonferroni correction data show the significant correlation between data. NA means not applicable.

3.5 Correlation between The Manifestations of Gastric Disease and Bacterial Virulence Factors

Based on the statistical analysis of multiplex-PCR results which were performed on 18 patients with ulcer and 62 patients with gastritis, no statistically significant difference was detected for the existence of *H. pylori cagA*, *hpaA*, *oipA*, *babA*, *vacAs1*, *vacAs2*, *vacAm1*, *vacAm2*, *ureA*, *ureB* and *dupA* virulence factor genes among patients with gastritis and ulcer (Table 3.1). *H. pylori cagA* gene was found in 60 % of all isolates; 61 % of patients with ulcer and 60 % of patients with gastritis. The *H. pylori vacA s1/m2* genotypes were the most frequent allelic combination of the *vacA* gene detected both among gastritis and ulcer patients. The *H. pylori oipA* gene prevalence was more frequent in ulcer patients than gastritis patients (50 %, 35%). Moreover, there was no statistically significant difference between the patients with ulcer and gastritis for the presence of *H. pylori hpaA* gene (89% in ulcer patients, 74 % for in gastritis patients),

for *babA* gene (22 % in ulcer patients, 15 % for in gastritis patients), for *ureA* gene (100 % in ulcer patients, 94 % for in gastritis patients) and for *ureB* gene (67 % in ulcer patients, 71 % for in gastritis patients). Additionally, no ulcer patients were colonized with *dupA* - positive *H. pylori* strains, and only 8 % of gastritis patients were colonized with *dupA* positive *H. pylori* strains. However, interestingly, the prevalence of *H. pylori napA* virulence factor was significantly higher in patients with ulcer than gastritis, which is consistent with previous studies (Satin *et al.*, 2000) (Table 3.1).



Table 3.1 : Comparison of virulence genes of *H. pylori* strains isolated from patients suffering from gastritis and ulcer. *H. pylori* strains with *napA* virulence factor gene are found more frequently in patients with ulcer than gastritis. Statistical significance was assessed with two-sided Fisher's exact test.

Virulence Genes Characteristic	Strain Type				P-value
	Ulcer		Gastritis		
	n	%	n	%	
<i>cagA</i>					
Absent	7	39	25	40	1,000
Present	11	61	37	60	
<i>hpaA</i>					
Absent	2	11	16	26	0,335
Present	16	89	46	74	
<i>oipA</i>					
Absent	9	50	40	65	0,285
Present	9	50	22	35	
<i>babA</i>					
Absent	14	78	55	85	0,475
Present	4	22	7	15	
<i>vacA s1</i>					
Absent	8	44	31	50	0,791
Present	10	56	31	50	
<i>vacA s2</i>					
Absent	15	83	55	89	0,686
Present	3	17	7	11	
<i>vacA m1</i>					
Absent	11	61	49	79	0,134
Present	7	39	13	21	
<i>vacA m2</i>					
Absent	9	50	17	27	0,090
Present	9	50	45	73	
<i>ureA</i>					
Absent	0	0	4	6	0,570
Present	18	100	58	94	
<i>ureB</i>					
Absent	6	33	18	29	0,774
Present	12	67	44	71	
<i>dupA</i>					
Absent	18	100	57	92	0,582
Present	0	0	5	8	
<i>napA</i>					
Absent	8	44	49	79	0,007*
Present	10	56	13	21	

3.6 Correlation between *H. pylori* Virulence Factors and Involvement of Th1, Th17 and Treg in *H. pylori*-Induced Gastric Diseases

The association of the Th1, Th17 and Treg cells with *H. pylori* infection has been explained (Larussa *et al.*, 2015), but the effect of *H. pylori* virulence factor on Th1, Th17 and Treg cells in infected patients either gastritis or ulcer has not been clearly understood. To determine whether there is an association between *H. pylori* virulence factors and Th1, Th17, and Treg cell responses, the expression levels of, IFN- γ , IL-17, and FOXP3 were examined, respectively in human gastric tissue specimens by quantitative RT-PCR (qRT-PCR). The significant fold change differences in gene expression level of IL-17, IFN- γ and FOXP3 were determined in patients with gastritis and ulcer, by calculating 95 % confidence intervals (CIs) of *H. pylori* negative control group. Patients with the confidence interval levels lower than the control group was accepted to have normal means with similar IL-17, IFN- γ and FOXP3 gene expression levels compared to control group. On the other hand, IL-17, IFN- γ and FOXP3 gene expression level higher patient samples with gastritis and ulcer, had higher 95 % confidence intervals (CIs) level than the control group (Figure 3.6).

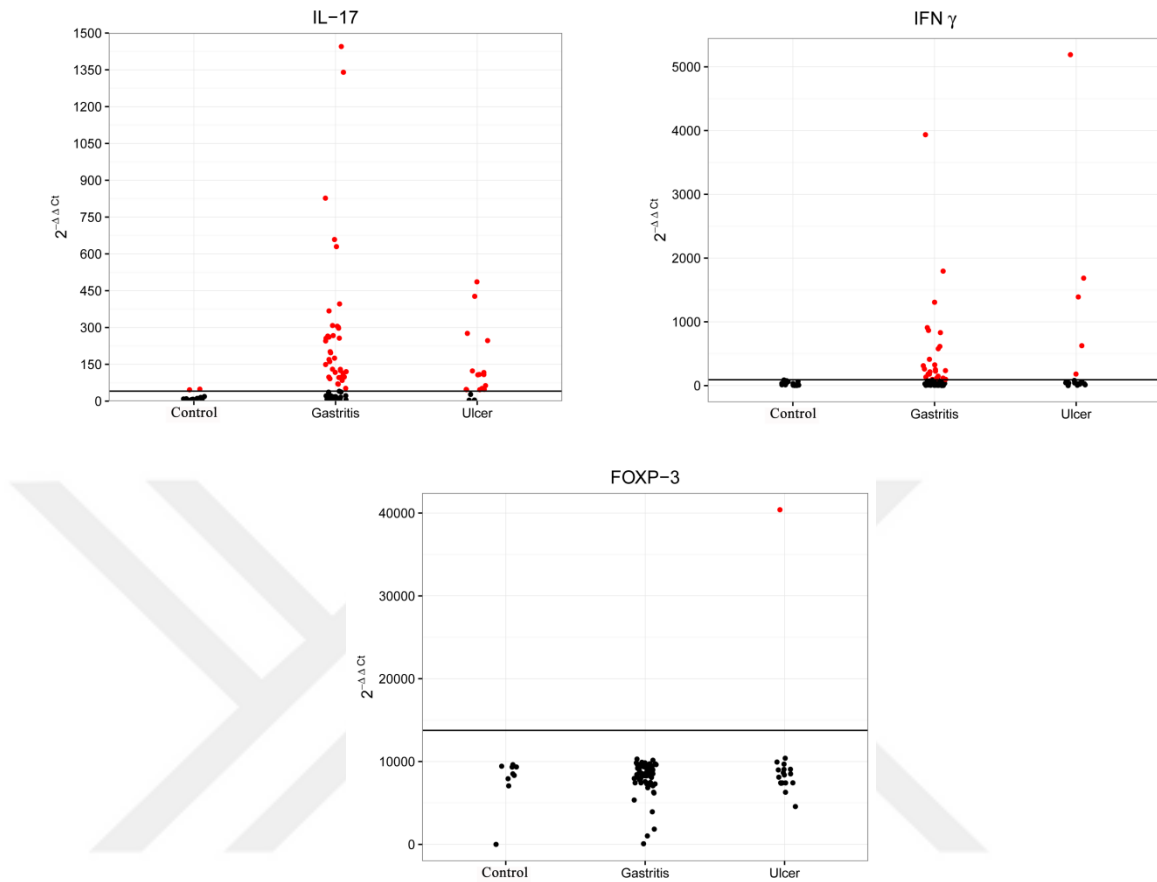


Figure 3.6 : Expression levels of IL-17, IFN- γ and FOXP3 in *H. pylori* infected ulcer and gastritis patients. IL-17, IFN- γ and FOXP3 positive samples according to the 95 % confidence intervals (CIs) level of control group are shown. 95 % confidence intervals (CIs) level of control group for IL-17 is 40, 50; IFN- γ is 91,58; FOXP3 is 13.768. The horizontal black lines indicate the 95 % confidence intervals values for each group. Dot plots were drawn with R program (N=62 for gastritis, N=18 for ulcer).

Based on the comparison of 95% confidence interval levels patients' qRT-PCR results with the control group that were negative for *H. pylori*, ulcer and gastritis patients infected with *H. pylori* showed mainly to have a Th17 response instead of Th1 and Treg response. 88% Th17, 33% Th1 and 5% Treg responses were observed in patients with ulcer and 80 % Th17, 35% Th1 and 0% Treg responses were observed in patients with gastritis. These results were supporting the association between Th17 response and *H. pylori* infection as previously indicated in the literature (Victoria Serelli *et al.*, 2012)

Then we assessed the potential correlation between T cell response and the *H. pylori*-specific virulence genes in patients with gastritis and ulcer using Pearson product-moment correlation coefficients method that is used to measure strength of a linear association between two variables in the data (Table 4). Gene expression data of cytokines and transcription factors from *H. pylori* -positive patients were normalized to *H. pylori* -negative patients. For the first time, we detected positive correlation between the *H. pylori dupA* virulence factor and IFN- γ in gastritis patients ($r=0.31$, $N=62$, $p<0.05$). Additionally, IL-17 gene expression was shown for the first time to significantly positively correlated with the *H. pylori babA* virulence factor in ulcer patients ($r=0.74$, $N=18$, $p<0.001$). These results suggested that correlation between *H. pylori* virulence factors and host immune responses could be used as a diagnostic prediction method for gastric diseases.

Table 3.2 : Correlation between virulence factors and cytokines, transcription factors. A Pearson’s correlation between virulence factors and cytokines were calculated using R (version 3.1.0, The R Foundation for Statistical Computing, Vienna, Austria; <http://www.r-project.org>).

Virulence Factors/Cytokines and Transcription Factors	Gastritis				Ulcer			
	IL-17	FOXP3	IFN- γ	ROR γ t	IL-17	FOXP3	IFN- γ	ROR γ t
<i>cagA</i>	0,21	-0,05	0,05	-0,02	0,17	-0,31	0,14	-0,34
<i>hpaA</i>	0,02	-0,02	-0,01	-0,09	0,27	0,04	0,15	0,17
<i>oipA</i>	-0,18	-0,09	0,07	0,06	-0,23	0,27	0,27	0,27
<i>babA</i>	-0,16	0,13	-0,13	-0,08	0,74	-0,11	-0,01	-0,15
<i>vacA s1</i>	0,13	-0,08	0,17	0,19	0,11	0,16	0,14	-0,16
<i>vacA s2</i>	0,02	0,09	-0,02	-0,08	-0,01	-0,08	-0,09	0,42
<i>vacA m1</i>	0,09	0,00	0,09	0,17	0,03	0,36	-0,14	0,28
<i>vacA m2</i>	-0,13	0,07	-0,06	-0,12	0,08	-0,26	0,22	-0,16
<i>ureA</i>	-0,15	-0,17	-0,01	-0,33	NA	NA	NA	NA
<i>ureB</i>	-0,12	0,02	0,05	-0,09	-0,20	0,10	-0,06	-0,40
<i>dupA</i>	-0,18	0,01	0,31	-0,04	NA	NA	NA	NA
<i>napA</i>	-0,07	0,02	-0,04	0,09	0,05	0,13	-0,38	0,10

3.7 Expert-Derived Models for Diagnostic Prediction of Gastric Diseases Using *H. pylori* Virulence Factors and Host Immune Responses

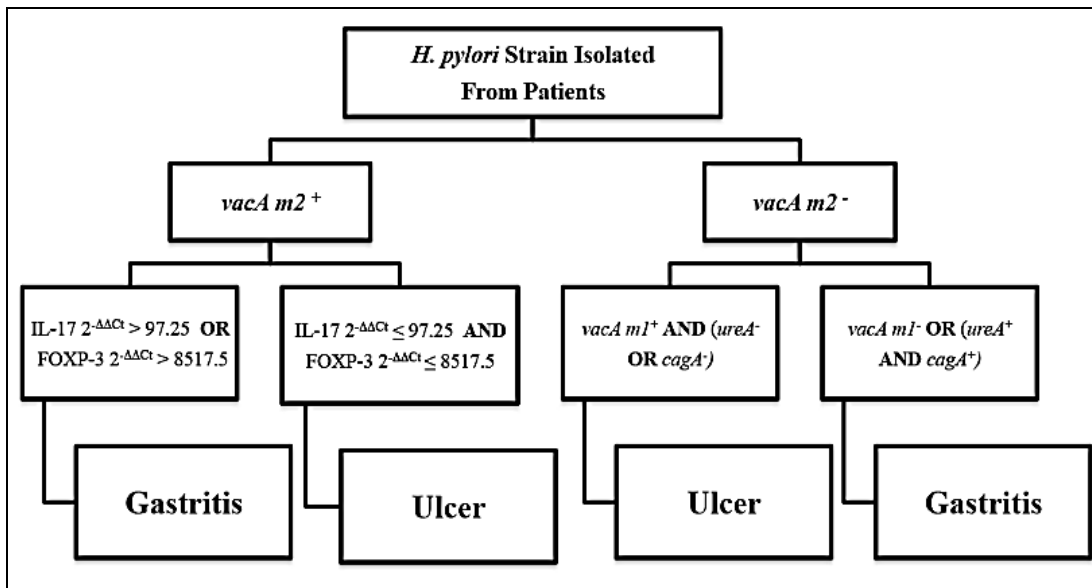
We created two expert-derived models (Figure 3.7 A and B), which show the relationship between the *H. pylori* specific virulence factors and Th1, Th17 and Treg response on the basis of their specific cytokines and transcription factor levels in *H.*

pylori infected gastritis or ulcer patients. Expert-derived models were built on a randomly selected training data consisting of two-thirds of the all dataset and performed by determining the most important feature first that can distinguish the ulcer and gastritis patients. With the guidance of expert-derived models knowledge of *H. pylori* virulence factors and host T cell response: *vacAm1/m2*, *cagA* and *ureA* presence or absence and IL-17, FOXP3 and IFN- γ expression level, it may be possible to predict a patient's clinical outcomes. It is possible to predict and detect significant differences with high accuracy between the patients with ulcer and gastritis in the expert-derived model in Figure 3.7 A.

Because of the low ulcer classification accuracy of the first model, (Figure 3.7 A) there was a need for additional factors to distinguish ulcer and gastritis patients more accurately. An additional factor (IFN- γ), which was added to the analysis in a second model eventually improved the ulcer classification accuracy from 44% to 61% (Figure 3.7 B).

Performance evaluation is made by repeated test set sampling to get comprehensive testing set enough to prevent large random fluctuations. Performance evaluation of the models showed that first model (Figure 3.7 A) has mean accuracy of 79% (standard deviation: 9%) in gastritis classification, 44% (standard deviation: 16%) in ulcer classification. Overall mean classification accuracy of this model was found to be 69% (Standard deviation: 8%). On the other hand, performance evaluations of the second model (Figure 3.7 B) with repeated test set sampling resulted in a performance of 71% (standard deviation: 1%) mean accuracy for gastritis predictions, 61% mean accuracy (standard deviation: 17%) for ulcer predictions, which corresponds to a 68% overall mean accuracy (standard deviation: 9%). Confusion matrix of models for best performing test set samples can be found in Table 3.3.

A



B

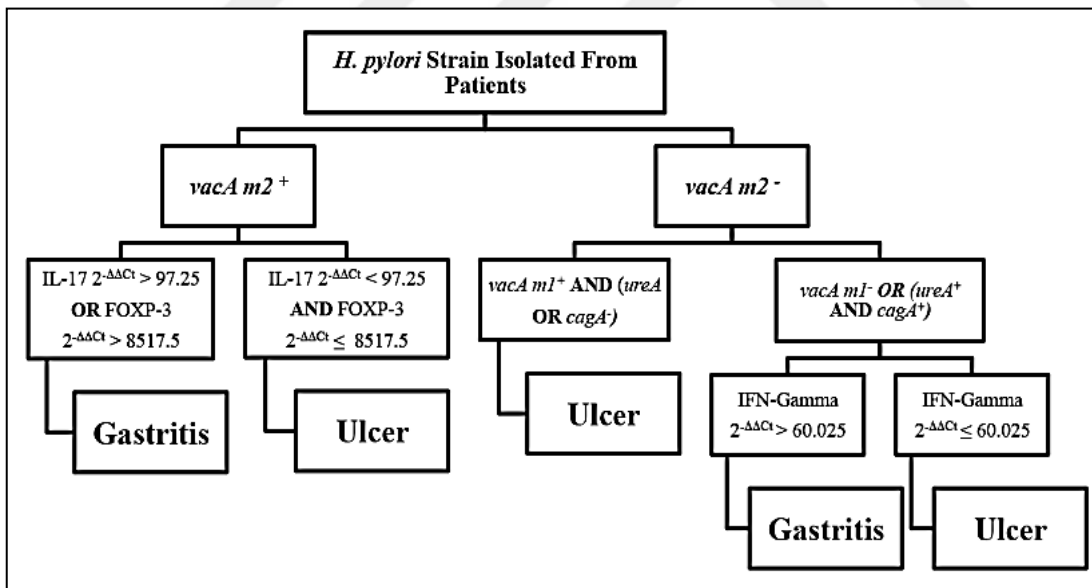


Figure 3.7 : Expert-derived models for diagnostic prediction of gastric diseases using *H. pylori* virulence factors and host immune responses. (A) shows that possible relationship prediction between the *vacAm1/m2*, *cagA* and *ureA* and IL-17, FOXP3 and patient's clinical outcomes and (B) shows that the possible relationship prediction between *vacAm1/m2*, *cagA* and *ureA* and IL-17, FOXP3 and IFN-γ and patient's clinical outcomes.

Table 3.3 : Confusion matrices for test set predictions. Test set samples with best overall classification accuracy was used in calculation of confusion matrices.

Based on Diagnosis/ Based on Model	First Model		Second Model	
	Actual Gastritis	Actual Ulcer	Actual Gastritis	Actual Ulcer
Predicted Gastritis	14	0	13	1
Predicted Ulcer	2	4	0	6

In the confusion matrix, the actual numbers were the numbers that represent the patients with gastritis and ulcer diagnosed based on clinical diagnosis. Additionally, the predicted numbers were the number of patients with ulcer or gastritis predicted by the models.

Although, these models may not yet be used for the diagnosis, they may give information about the prognosis of the diseases. Gastric malignancies may initiate with gastritis and progress to ulcer and at certain cases to gastric cancer. These models help to predict this progress. Furthermore, these models will assist to understand the mechanism of the gastric diseases with the help of genetic diversity of bacteria and the host immune responses and will be advantageous in predicting the clinical presentation of an *H. pylori* infection.

3.8 The ‘on/off’ Functional Status of *oipA* Gene

CagA and OipA virulence factors of *H. pylori* are involved in the ability of bacteria to colonize the gastric mucosa layer and to regulate the host environment during the development of chronic infection. In the literature, it has been suggested that the presence or absence of *cagA* gene and functional status of *oipA* gene have been involved in the induction of cellular transformations which plays an important role in the development of gastritis (Torres *et al.*, 2014). To investigate the relation between the ‘on/off’ functional status of *oipA* gene and presence or absence of *cagA* virulence factors on gastritis, PCR and sequencing were performed to 5 patient samples with gastritis as a preliminary study. To assess the *oipA* frame status by the CT repeat numbers on the signal peptide coding region, sequencing results of the 5 samples with gastritis have been evaluated. The OipA protein coding gene, *oipA* gene, is regulated by a switch (like ‘on’ or ‘off’ patterns) by changing the number of CT dinucleotide repeats

in the signal-peptide coding region of this gene. When there is 6, 8 (3+6) or other CT dinucleotide repeats that keep the peptide in frame that means *oipA* gene is “on” status. Otherwise, 7 and 5 CT repeats means the status of *oipA* gene is “off”, which is nonfunctional *oipA* gene (Table 3.4). A PCR-based methodology was applied to detect presence of CagA virulence factor in samples. It was shown that patients infected with *H. pylori* with non-functional *oipA* gene were also negative for the CagA virulence factor. This preliminary data suggest that there is a close link between the *H. pylori* CagA virulence factor and *oipA* gene status in our patients with gastritis, which is consistent with the literature (Torres *et al.*, 2014). Further studies and analysis with a larger number of samples are needed to explain this relationship between *oipA* gene status and other *H. pylori*-specific virulence factors on both gastritis and ulcer.

Table 3.4 : Sequence of the signal peptide coding region of *H. pylori* positive gastritis patients. Analysis in G27 strain was performed as a positive control.

Sequence of Signal Peptide Encoding Region of Oip A	CT Number	Number of Patients
ATG AAA AAA GCT CTC TTA CTA ACT CTC TCT CTC TCG TTC TGG CTC Met K K A L L L T L S L S F W L	6 (on)	P1
ATG AAA AAA GCT CTC TTA CTA ACT CTC TCT CTC TCT CGT TTT GGC TCC Met K K A L L L T L S L S R F G	7 (off)	P2
ATG AAA AAA GCT CTC TTA CTA ACT CTC TCT CTC GTT TTG GCT CCA Met K K A L L L T L S L V L A	5 (off)	P3
ATG AAA AAA GCT CTC TTA CTC TCT CTC TCT CTC TCG TTC TGG CTC CAC Met K K A L L L S L S L S F W L	8 (3+6)(on)	P4
ATG AAA AAA GCT CTC TTA CTC TCT CTC TCT CTC TCG TTC TGG CTC CAC Met K K A L L L S L S L S F W L	8 (3+6)(on)	P5
ATG AAA AAA GCT CTC TTA CTA ACT CTC TCT CTC TCG TTC TGG CTC Met K K A L L L T L S L S F W L	6 (on)	G27

4. DISCUSSION

The virulence factors of *H. pylori*, which are known to be directly correlated with the extreme degree of genetic heterogeneity in *H. pylori* genome, play a pivotal role in determining the outcome of *H. pylori* infection. The immune response of the host including T cell activation has also been the subject of recent studies, together with specific virulence factors of *H. pylori* (Kaebisch *et al.*, 2014).

Multiplex-PCR based genotyping systems have initially been developed to detect the *H. pylori*-specific virulence factors. However, these assays were not sufficient to detect many *H. pylori*-specific virulence factors that are thought to be associated with bacterial pathogenesis and increase the risk for developing severe clinical manifestations, in the same PCR assay. In this study, a genotyping system-based multiplex-PCR assay was developed to detect nine potential virulence genes (*vacA*, *cagA*, *oipA*, *babA*, *hpaA*, *dupA*, *napA*, *ureA*, *ureB*) in three PCR assays directly from human gastric biopsies. This assay is able to detect both the presence and absence of *H. pylori* by *ureA* and *ureB* genes, and identify the leading disease-associated virulence genes of *H. pylori* strains isolated from patients. Moreover, this PCR assay helps to better understand the relationship between the virulence factors and different clinical forms of gastric lesions and the pathogenesis of each of these factors.

Multiplex-PCR assay results do not indicate any correlation between the *H. pylori* genes *vacA*, *cagA*, *oipA*, *babA*, *hpaA*, *dupA*, *ureA*, *ureB* and *H. pylori*-related gastritis and ulcer diseases. However, a statistically significant relationship ($p=0.007$) between *napA* gene and *H. pylori*-associated ulcer disease were identified, as reported previously (Rautelin *et al.*, 1993).

Virulence factors of *H. pylori* are one of the major determinants of *H. pylori*-related infection and disease course. Association between the virulence factors can be another

determinant of *H. pylori*-related gastric diseases. Multiplex-PCR assay results indicate a positive correlation between the CagA-HpaA and NapA-VacA s2 virulence factors for patients with gastritis and a negative correlation between the VacA m1-VacA m2 virulence factors for patients with gastritis and ulcer. Negative correlation between the VacA m1-VacA m2 for both patients with gastritis and ulcer can be explained by the allelic variation in the mid-region of the *vacA* gene sequence. Variations in *vacA* gene sequences are mainly found restricted to three genetic regions: the signal sequence region (*s*-region), mid-region (*m*-region) and the recently identified intermediate-region (*i*-region). Also, two types of allelic variations in the *s*-region and *m*-region are classified as *s1* or *s2* and *m1* or *m2*. Isolated *H. pylori* strains include one of the allele of *s*-region (*s1* or *s2*) and one of the allele of *m*-region (*m1* or *m2*). Adhesion to the gastric mucosa is a critical initial step in the *H. pylori* pathogenesis. The virulence factors play role in the bacterial adhesion. Positive correlation between CagA-HpaA and NapA-VacA s2 may be explained by the role of these virulence factors on the bacterial adhesion and also colonization on the gastric mucosa. Experimental results of this study suggest that correlation between virulence factors would be a useful marker to identify patients who are at higher risk for specific *H. pylori*-related gastric diseases. However, a large sample size would provide a more precise estimate of the association between these virulence factors and *H. pylori*-related gastric diseases.

There are many different tests available to identify *H. pylori*, each of which has certain advantages and disadvantages. However, due to poor sensitivity or specificity, none of them can be considered as gold standard. Rapid urease test and pathological staining are the most useful methods in the clinical practice to detect *H. pylori*. In this study, multiplex urease PCR assay was developed to detect *ureA* and *ureB* virulence genes of *H. pylori*. Comparison of the results of the rapid urease test with histopathological staining and the multiplex urease PCR indicated that detection rate of *H. pylori* with multiplex urease PCR and histopathological staining is higher than the rapid urease test. The possible reason for the lack of sensitivity of rapid urease test might be the region of biopsy specimens that is taken to be only from the antrum part of the stomach where the most of the bacterial colonization can be seen. However, localization of *H. pylori* colonies in patients can vary as being only in antrum, only in corpus or in both antrum

and the corpus part of the stomach. In the multiplex urease PCR and histopathological staining method two biopsy specimens, one from antrum and one from corpus part of stomach are taken to increase the detection rate of *H. pylori*. Additionally, we identified that sensitivity of multiplex urease PCR assay was higher than the histopathological staining in this study. The reason for this may be because PCR may detect the DNA of the bacteria that has just begun to colonize and the histopathological staining may not detect this newly colonized bacteria. Using the combinations of rapid urease test, histopathological staining and multiplex urease PCR assay, may provide quite satisfactory results in the diagnosis of *H. pylori* infection.

The type of host immune response, particularly driven by T cells, is crucial for the outcome of *H. pylori* infection in humans. Given the increasing number of reports, correlations between the characteristics of the T helper cell responses of Th1, Th17, and Treg cells and virulence factors are great interest in determining the outcomes of *H. pylori* infections (Aujla *et al.*, 2007; Larussa *et al.*, 2015). In previous studies, it was shown that Th1 cells contributed to inflammation and participated in the pathogenesis of *H. pylori* infection (Shi *et al.*, 2010). The role of Th17 in host protection against gram-negative bacteria has also been shown (Smythies *et al.*, 2000). However, the role of Th17 has not been clearly explicated. In this study, we characterized the cell responses of T helper cells, particularly Th1 and Th17, in *H. pylori* positive clinical isolates obtained from patients with gastritis and ulcer. One of the most prominent outcomes of the study was the identification of a higher Th17 cell response compared to Th1 response against *H. pylori* infection in patients with gastritis and ulcer. This finding has been shown previously in mice models (Aujla *et al.*, 2007). To the best of our knowledge, this is the first time that a higher Th17 cell response compared to Th1 response to *H. pylori* infection has been shown in clinical samples. A possible reason for this outcome is that, Th17 cells may play a role in the pathogenesis of *H. pylori* infection by promoting the mucosal inflammation and thus contributing to bacterial colonization. Also Th1 cells causes mucosal inflammation in *H. pylori* infection, but our results suggested that Th17 cell responses may be induced earlier than Th1 cell responses in *H. pylori* infected ulcer and gastritis patients. To obtain more definitive results about the induction of Th17 cell responses in *H. pylori* infected ulcer and gastritis patients, further

studies including larger number of clinical samples should be done. Moreover, ELISA-based assays for quantifying the levels of secreted proteins can be performed.

The *H. pylori* specific virulence factors are important for the pathogenesis of this organism. These virulence factors facilitate the survival of the bacterium and modify the immune response. In support of this, we found a correlation between *dupA* positive strains and IFN- γ (Th1-specific cytokine) expression levels in patients with gastritis. Our results confirmed a previous study that suggests *dupA* causing gastric inflammation and pathology either directly or indirectly over action upon infiltrating leukocytes rather than upon epithelial cells. It was also referred in the study that *dupA* positive *H. pylori* strains increase the risk of disease by stimulating a more definite Th1 response (Hussein *et al.*, 2010).

Virulence factor *babA* helps bacteria to adhere to the fucosylated Lewis b antigen on the surface of the gastric epithelial cells. *H. pylori* adheres to the host and secretes effector molecules that can change function and viability of gastric epithelial cells. The production of various cytokines, gastric inflammation, and epithelial cell damage is enhanced by these changes. Also recent studies have indicated that there is severe mucosal injury in patients infected with *babA* positive *H. pylori* strains (Ohno *et al.*, 2011). Our data showed, for the first time, a relationship between *babA* positivity and interleukin-17 (IL-17) expression levels in patients with ulcer.

In this present study, it was identified that there are significant correlations between the virulence factors of *H. pylori*, responses of T cells (Th1, Th17, Treg) and clinical outcomes of *H. pylori* infections, and these eventually helped evolution of expert-derived models. Initially, first model was developed using the strategy outlined in the method section. To improve the low classification accuracy of ulcer disease in this model, we added additional factors to the model. The addition of IFN- γ to the second model (model 2) eventually improved the classification accuracy of ulcer from 44% to 61% with a cost of only one misclassified gastritis patient. On overall, to our knowledge, this is the first report of expert-derived models, which points out the relationship between *H. pylori* virulence factors and host immune responses. Despite a need to improve the accuracy, these models could contribute to the prediction of clinical

outcomes of a patients infected with *H. pylori* by screening the patient's *H. pylori vacA m1/m2, ureA and cagA* status and IL-17, FOXP3 and IFN- γ expression levels.

OipA is one of the *H. pylori* specific outer membrane proteins that takes an important role in bacterial pathogenicity such as virulence (Torres *et al.*, 2014). Expression of *oipA* gene is regulated by CT dinucleotide repeats in the 5' signal coding regions of the gene. DNA sequence analysis and PCR based findings suggested an association between the on status of *oipA* and presence of the CagA virulence factor on gastritis. Oip A virulence factor has an important role in the bacterial adhesion and CagA is encoded by the *cag* Pathogenicity Island that encodes for type IV secretion system used to inject CagA virulence factor into the target cell upon bacterial attachment. Moreover, it has been known that Cag A positive strains are associated with severe clinical outcomes. The association between *cagA* and *oipA* may be explained by their role in the bacterial attachment to the gastric layer, since it is known that adherent *H. pylori* strains are more prone to elimination by the host immune system. Correlation between the Cag A virulence factors and functional status of *oipA* may present a putative marker for gastric diseases. To obtain more definitive data, this study has to be applied to a larger sample set with gastritis and ulcer.

In conclusion, these data support the hypothesis that the relationship between the *H. pylori* specific virulence factors and Th1, Th17 and Treg cells have an important role in the progression of *H. pylori* driven pathology. A better understanding of the effects of virulence factors on T cell responses may help to estimate the clinical outcomes of *H. pylori* infection and develop therapies to prevent risk for gastric cancer development. Correlation between the virulence factors may be additional criteria that help to estimate the *H. pylori* related gastric diseases.



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APPENDICES

APPENDIX A : Laboratory Equipment

APPENDIX B : Chemicals and Kits

APPENDIX C : Preparation and Composition of Buffers and Solutions



APPENDIX A

Laminar Air Flow Cabinets	FASTER BH-EN 2003 Precisa BJ 610C
Pipettes	10 µL, 20 µL , 100 µl, 200 µL, 1000 µl Socorex And 10 µL, 100 µl, 1000 µl Biohit
Centrifuge	Beckman Coulter Allegra™ 25R Centrifuge Scanspeed 1730 R Labogene Scanspeed mini
Gel Documentation System	UVI PhotoMW Version 99.05 for Windows ChemiDoc™ MP Imaging System ORTE Observable Real Time Electrophoresisi, SALUBRIS
SpeedMill PLUS Homogenizator	Analytikjena
Thermal cycler	Applied Biosystems
Vortex	Mixer Uzusio VTX-3000L, LMS
Quick spin	LMS
Ice Machine	Scotsman AF10
Freezers	Altus (+4 °C) Siemens (-20 °C) Haier (-80 °C)
Power Supply	BIO-RAD
Centrifuge tubes	Interlab
Eppendorf tubes	Interlab
Nanodrop 2000	Thermo Scientific
Examination gloves	Beybi

APPENDIX B

Agarose	Appli Chem
Glacial Acetic Acid	Appli Chem
dNTP	Thermo
EDTA	Appli Chem
SYBR Gold	Fermentas
Tris Base	Sigma-Aldrich
RNA Stabilization Solution	Sigma-Aldrich
Power SYBR Green PCR Master Mix	Applied Biosystems
5x FIREPol® Master Mix Ready to Load	Solis Biodyne
Quick g-DNA™ 130	Zymo Research
Innu Prep RNA Isolation Kit	Analytikjena
cDNA Synthesis Kit	Applied Biosystems

APPENDIX C

TAE (Tris-Acetate-EDTA) Buffer (50X)

Tris base	242 g
Glacial Acetic Acid	57.1 ml
EDTA	100 ml (0.5 M, pH 8.0)

Add ddH₂O to 1 liter and adjust the pH to 8.0

Mini Agarose Gel (1%)

Agarose	0.5 g
TAE buffer (1X)	50 mL

CURRICULUM VITAE



Name Surname : Sinem ÖKTEM OKULLU

Place and Date of Birth : Istanbul, 25.04.1984

Address : Ulubatlı Hasan Cd. Mimar Sinan Mah. No:117 D:7
Çekmeköy/İstanbul

E-Mail : sinemoktemokullu@gmail.com

B.Sc.: Abant İzzet Baysal University, Science & Letters Faculty, Department of Biology, 2007

M.Sc.: Yeditepe University, Faculty of Engineering and Architecture, Department of Genetics and Bioengineering, 2009

PROFESSIONAL EXPERIENCE AND REWARDS

Thesis Awards: Technology Development Foundation of Turkey, 2009 Thesis Award By Observable Real Time Electrophoresis

PUBLICATIONS AND PRESENTATIONS PRODUCED FROM THESIS

- **Oktem-Okullu, S.,** Tiftikci, A., Saruc, M., Cicek, B., Vardareli, E., Tozun, N., Kocagoz, T., Sezerman, U., Yavuz A. S., Sayi-Yazgan, A. (2015). Multiplex-PCR-Based Screening and Computational Modeling of Virulence Factors and T-Cell Mediated Immunity in *Helicobacter pylori* Infections for Accurate Clinical Diagnosis. *PloS One*, 10(8):e0136212. doi: 10.1371.
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