Bora Kazım BÖLEK

M.S. Thesis In Biology

July - 2006

DNA SEQUENCE ANALYSIS OF THE CAGA 3' GENE OF HELICOBACTER PYLORI STRAINS IN TURKEY

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APPROVAL

I certify that this thesis satisfies all the requirements as a thesis for the degree of Master of Science.

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This is to certify that I have read this thesis and that in my opinion it is fully adequate, in scope and quality, as a thesis for the degree of Master of Science.

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ABSTRACT

Recent application of multiplex PCR for genotyping *Helicobacter pylori* strains directly from biopsies revealed variable results (detection of amplicons from DNA extracted by boiling biopsies, variable size amplicons and deletions, uniform intensity of amplicon bands). We aimed to look at how applicable the technique is for determining cagA and vacA genotypes and to correlate the results with the severity of the disease. Method: *H. pylori* isolates from 52 patients (35 duodenal ulcer (DU), 7 gastric ulcer (GU) and 10 gastritis) were included .Three antral biopsies were obtained for CLO and PCR. Primers for cagA, vacA s1s2, and m1m2 alleles were used. Results: No PCR amplicons were obtained from boiling biopsies, thus DNA was extracted by QIAamp kit. *H. pylori* was positive in 84.6% of the patients (85.7% DU, 100% GU, 70% gastritis). The cagA gene was detected in 86.6% DU, 71.4% GU and 57.0% gastritis patients. The vacA allele distribution among cagA-positive strains was 80.7% vacA s1m1 in DU and 60.0% in GU patients while gastritis patients showed 75.0% s1m2 genotype. There was a significant correlation between *H. pylori* cagA+ vacA-s1m1 genotype and peptic ulcers. No variability in the amplicon sizes was found and the intensity of the amplicon bands was not uniform. A deleted band of approximately 420bp below the m1 band was detected in strains from 2 DU and 1 GU patient. The multiplex PCR although rapid and an effective tool for detecting several genes in a single step system one has to adjust for optimization of the technique when genotyping *H. pylori* direct from biopsies.

Keywords: *Helicobacter pylori*, cagA, vacA, duodenal ulcer, gastric ulcer, gastritis, multiplex PCR.

TÜRKİYE'DEKİ HELICOBACTER PYLORI CAGA 3' GENİNİN DNA DİZİ ANALİZİ

Bora Kazım BÖLEK

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ÖZ

Günümüzde, mide biopsilerinde bulunan *Helicobacter pylori* suşlarının genotiplendirilmesi için kullanılan multipleks PZR yöntemiyle şu zamana kadar farklılık arz eden sonuçlar elde edilmiştir (kaynatma yöntemiyle DNA ve PZR ürünü elde edilmesi, farklı uzunluklarda PZR ürünleri ve delesyonların bulunması, farklı yoğunluklarda PZR ürün bantlarının tespit edilmesi). Bu çalışmayla, kullandığımız tekniğin cagA ve vacA genotiplerini belirlemede ve hastalık sonuçlarıyla ilişkilendirilmesinde ne derece faydalı olduğunu bulmayı amaçladık. Metod: 52 hastadan izole edilen *H. Pylori* (35 duodenum ülseri (DÜ), 7 mide ülseri (MÜ), 10 gastrit hastası) incelemeye tabi tutuldu. CLO ve PZR için her hastanın antrumundan 3 adet biopsi alındı. CagA, vacA s1s2 ve m1m2 bölgelerine özel 3 set primer kullanılarak multipleks PZR yapıldı. Sonuç: Kaynatma yöntemiyle hiçbir PZR ürünü elde edilemediği için QIAamp kiti kullanılarak DNA izolasyonu yapıldı. Hastaların %84.6'sı *H. pylori* pozitif olarak tespit edildi (DÜ hastalarının %85.7'si, GÜ hastalarının %100'ü ve gastrit hastalarının %70'i). CagA geni DÜ hastalarının %86.6, GÜ hastalarının %71.4 ve gastrit hastalarının %57'sinde tespit edildi. VacA allelinin cagA pozitif suşlarda görülme oranı ise şöyleydi: VacA s1m1 DÜ hastalarında %80.7 ve GÜ hastalarında %60 oranında tespit edildi. Gastrit hastalarının %75'i ise s1m2 genotipine sahipti. *H. pylori* cagA pozitif vacA-s1m1 genotipi ile peptik ülser hastalıkları arasında önemli bir ilişki bulunmaktadır. PZR ürün bantlarının uzunluklarında herhangi bir farklılık görülmemekle beraber bant yoğunlukları birbirinden farklıydı. 2 DÜ ve 1 GÜ hastasının vacA m1 allelinde yaklaşık 420 baz çifti uzunluğunda, delesyona uğramış bölge saptandı. Multipleks PZR yöntemi çeşitli genlerin tek seferde hızlı ve etkili bir şekilde belirlenmesinde kullanılan bir yöntem olmasına rağmen bu yöntemin *H. Pylori* genotip analizinde optimize edilmesi gerekmektedir.

Anahtar Kelimeler: *Helicobacter pylori*, cagA, vacA, duedonum ülseri, mide ülseri, gastrit, multipleks PZR.

DEDICATION

To my beloved ones

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LIST OF SYSMBOLS AND ABBREVIATIONS

SYMBOL/ABBREVIATION

bp	Base pair
cagA	Cytotoxin-associated gene A
CLO	*Campylobacter* like organism
dNTP	Deoxynucleoside triphosphate
DU	Duodenal ulcer
EDTA	Ethylenedinitrilo tetraacetate
G	Gastritis
GU	Gastric ulcer
IS	Insertion sequence
kb	Kilobase
kDa	Kilodalton
m	Middle region
M	Molar
MALT	Mucosa associated lymphoid tissue
µl	Microliter
PAI	Pathogenicity island
PCR	Polymerase chain reaction
pmol	Picomole
PUD	Peptic ulcer disease
rpm	Rotation per minute
s	Signal region
TBE	Tris-Boric acid-EDTA
vacA	Vacuolating-associated gene A

CHAPTER 1

INTRODUCTION

Helicobacter pylori infection is widely distributed worldwide. It causes chronic gastritis and peptic ulcer diseases (PUD) and plays an important role in gastric carcinoma and mucosa associated lymphoid tissue (MALT) lymphoma [1]. The bacterium was isolated by Marshall and Warren from the human stomach in 1983 [97]. In the developing countries, 70-90% of the populations are infected while in the developed countries the prevalence of infection is much lower [1]. However, most people who harbor *H. pylori* are asymptomatic, and only a few percentages of infected patients develop PUD and still fewer may progress to gastric cancer [98]. This may be because of some strains of *H. pylori* being more virulent than others [99]. The variability of clinical manifestations is associated with several factors such as strain virulence factors, host immunological factor and environmental influences, or their combinations [100].

Of the several *H. pylori* virulence genes that may play a role in pathogenesis, the cagA gene located in the cag pathogenicity island (cag PAI) is possessed by approximately 68% of the strains [42, 71] and the vacA gene is virtually present in all strains [24]. The effects of these two major virulence factors on gastric epithelial cells illustrate the complex network of cellular interactions activated by the bacterium [114]. Patients with duodenitis, duodenal ulcers, and gastric ulcers are mostly infected with cagA-vacA positive strains suggesting that cagA and the co-expressed cytotoxin play an important role in pathogenesis [16]. *H. pylori* genotypes are extremely diverse, due to point mutations, substitutions, insertions and/or deletions in their genomes and cancer risk is believed to be related to *H. pylori* strain differences [31].

From the few studies conducted on *H. pylori* genotyping of virulence genes using multiplex PCR technique, Chisholm et al. [101] had recently introduced the one-step system for genotyping of vacA alleles. Another study applied the technique for genotyping *H. pylori* in stool samples but required modifications in the DNA extraction and the number of amplification cycles [102]. In another study multiplex PCR was performed on DNA extracted from formalin fixed paraffin wax embedded gastric tissue sections [121]. Monstein et al. [25] reported an unexpected multiplex PCR patterns that showed the presence of different sizes of vacA amplicons. More recently Chattopadhyay et al. [113] showed the detection of vacA and cagA genotypes by multiplex PCR using DNA extracted from biopsies by boiling in water bath. No size difference was detected in their amplicons but they reported for the first time the detection of a deletion in the vacA m1 allele in one of the strains examined and also the colonization of multiple *H. pylori* strains.

As indicated above multiplex PCR technique has allowed simultaneous amplification of several *H. pylori* virulent genes directly from biopsies. However the differences in the results obtained and the various approaches used in these studies (DNA extraction by boiling biopsies, detection of variable sizes of vacA amplicons and deletions, multiple colonization) were considered unique since they were not reported earlier in any two-step PCR studies done on DNA extracted from biopsies. We have conducted this study to apply the technique on our strains to look at the genotype pattern and whether or not such differences will also be encountered and to correlate the results with the severity of the disease.

CHAPTER 2

REVIEW OF LITERATURE

2.1 *HELICOBACTER PYLORI* VIRULENCE FACTORS

Various virulence factors are possessed by *H. pylori* such as flagella, urease, heat shock protein, cytotoxin associated gene (cagA) of the cag PAI and the vacuolating cytotoxin (vacA) gene, which play a significant role in the pathogenesis [1]. The powerful urease enzyme breaks down urea into ammonia and bicarbonate thereby helping neutralize the acidity of stomach and allow the initial colonization [2,3].

2.2 PATHOGENESIS

The cytotoxin associated gene (cagA) encodes the CagA protein of 120-145 kDa [4]. It was first described as a virulence factor of *H. pylori* that is associated with peptic ulcers [5]. Since specific antibodies against CagA are detected in sera of virtually all infected individuals it is obvious that CagA is quite immunogenic [6]. The VacA protein produced by the vacA gene is an 87 kDa toxin [7,8,9]. It induces the formation of intracellular vacuoles, loss of epithelial homeostasis and disruption of epithelial barriers. Potential pathways by which VacA causes epithelial barrier disruption include apoptosis, depolarization of the membrane potential, increased permeability of tight junctions and decreased transepithelial electrical resistance, permeabilization of the apical plasma membrane to urea and bicarbonate, and detachment of epithelial cells from the basement membrane [10,11,12]. Although all *H. pylori* strains possess the vacA gene only 50-60% exhibit detectable cytotoxin activity [13]. Once attached to the gastric epithelial cell, *H. pylori* may induce some damages by producing ammonia and causing vacuolation. Benign forms of cytotoxins may be associated with gastritis in

asymptomatic subjects or non-ulcer subjects whereas more aggressive forms are likely to be associated with peptic ulcer [14].

H. pylori strains are classified as type 1 (vacA-positive and cagA-positive) and type 2 (strains that don't possess cagA gene and don't express either the CagA protein or the VacA protein) [15]. Patients with duodenitis, duodenal ulcers, and gastric ulcers are those that are mostly infected with type I strains, which suggests that cagA and coexpressed cytotoxin play a role in its pathogenicity [16].

The presence of cagA has been significantly associated with duodenal ulceration, gastric mucosal atrophy, and gastric cancer [6,17,18], although some studies denied this association [19]. Similarly several studies have also shown that the presence of vacA is associated with PUD [9].

A key feature of *H. pylori* is the enormous genomic micro-diversity of its strains distributed worldwide, even many individuals appear to be infected by a unique strain. At the same time, different countries seem to be characterised by a particular geographic pattern of different *H. pylori* genotypes [20,21]. In Western populations, gastric atrophy, duodenal ulceration, intestinal metaplasia, gastric carcinoma are more common among patients infected with cagA positive than among patients infected with cagA negative strains [16,20]. Yet, in China and Japan the association between cagA positivity and virulence of *H. pylori* strains was equally frequent among both diseased and control patients [20]. It was shown that in East Asia and in the Western countries, distinct variants of *H. pylori* cagA genes were associated with particular vacA subtypes [22].

2.2.1 cag Pathogenicity Island (cag PAI)

The 40-kb DNA insertion segment of *H. pylori* genome known as the cag PAI has been acquired from other bacteria. The cag PAI contains 31 putative genes, 6 of which are thought to encode components of a bacterial type IV secretion system, which specializes in the transfer of a variety of multimolecular complexes across the bacterial membrane to the extracellular space or into other cells [16,41,42].

The cag PAI region is flanked by 31 bp direct repeats, insertion sequences that may be present in multiple copies and a high density of genes are packed in this region

[40]. According to previous studies, cag PAI can be found either as a single uninterrupted unit divided into two regions, namely cag I and cag II by an insertion sequence called IS605 or by a large piece of chromosomal DNA or partially deleted [21,46,47]. Usually, the insertion sequences are characterized by the presence of a single transposase flanked by inverted repeats [47]. Partial or complete deletions in the cag PAI can be in strains associated with benign gastric pathology [16,48,49].

The cagA gene found within the cag PAI is present in 50–70% of *H. pylori* isolates from Western populations, and in 90% or more of the strains in Eastern populations [43,74,75,108]. In clinical studies and animal infection experiments, type-I strains, which possess the cag PAI genes, were responsible for severe gastric diseases, such as peptic ulcer disease and gastric malignancies [44,45].

The cag PAI encodes a functional type IV secretion apparatus homologous to the VirB/D4 type IV secretion system (T4SS) of the plant pathogen *Agrobacterium tumefaciens* [50,51]. The T4SS encoded by the cag PAI of *H. pylori* is responsible for the translocation of the protein CagA into the host cells. Once translocated, CagA is tyrosine-phosphorylated by Src kinase and it interferes with phosphorylation events of the normal signaling cascades within the host cell [52,103]. Interaction of the T4SS apparatus and of the bacterium with the host cell-surface appears to be sufficient to trigger the production of pro-inflammatory cytokines, including IL-8 [53]. Only a fraction of the 29 putative proteins encoded by *H. pylori* cag PAI have been recognized to be *A. tumefaciens* VirB/D4 structural or functional homologues [54]. Some of them play a structural role in the assembly of the secretion machinery, including CagT, CagV, CagW, CagX and CagY, which are homologues of VirB7, VirB8, VirB6, VirB9 and VirB10, respectively [55,56].

The cag PAI has also been implicated in the induction of interleukin-8 (IL-8) in cultured gastric cells. As shown by the systematic mutagenesis studies of individual genes encoding the cag PAI, there are at least 17 of 27 genes that are found to be essential for translocation of cagA into host cells (syringe-like function), and 14 were necessary for *H. pylori* to fully induce transcription of IL-8 [57]. This property contributes to the proinflammatory power of the strains and thus also to its virulence capability. Nevertheless, the induction of IL-8 is not exclusively linked to cag PAI.

Exposure of gastric epithelial cells to cag PAI-positive *H. pylori* can activate the proto-oncogenes c-*fos* and c-*jun*, a crucial step in the development of *H. pylori*-related neoplasia [58]. An intact cag PAI has therefore been thought to contribute toward full proinflammatory power of *H. pylori*. However, the island has proved to be prone to disruption due to various genetic rearrangements occurring within and outside the constituent genes. Further still, the impact of intactness or rearrangement of the island on the progression of gastroduodenal pathology has been debated. It has been argued that abolition of cagE gene results in a considerable reduction of IL-8 production [16,41,59,60,61,62] whereas strains capable of eliciting an IL-8 response irrespective of an intact cag PAI have been described [63,64]. It was also indicated that the IL-8 response was found to be independent of the cag PAI, since cag PAI-deficient *H. pylori* stimulated IL-8 production [106]. Some strains causing nonulcer dyspepsia have regions of the promoter of cagA gene deleted [64], although this gene has long been regarded as a marker for the functionality of the cag PAI. However, due to rearrangements often inhibiting cagA, it does not seem to be a reliable indicator of the virulence spectrum of strains [65].

The cagG gene was shown to be a better indicator for the presence of an intact cag PAI than the cagE gene in French isolates [66]. Deletion of cagE, cagT, cagA, cagG, and cagM genes was reported in all cases of chronic gastritis, gastric ulcer and gastric cancer, indicating that pathogenicity of *H. pylori* might not be determined by cag PAI genes alone in such cases [67]. In several patients from Taiwan, the presence of cagA, cagC, cagE, cagF, cagN and cagT genes in the cag PAI showed no relationship to the type of disease and/or the histological features present in the patients [68]. The involvement of these genes and others in eliciting a strong immune response has also been contested [69]

It has been reported that the elements that appeared to be the best markers for the presence of cag PAI were the picB and the virD4 genes. Because the cag PAI may be partially deleted or diversely organized, it is likely that the presence of one or even several genes is not sufficient to assess the presence of this region [46].

2.2.2 Cytotoxin Associated Gene A (cagA)

The cagA gene is present downstream of the cag PAI located at the 3' end of the glutamate racemase gene [16]. The cagA gene encodes the CagA protein that has a molecular weight of 120–140 kDa and it is considered as a marker for virulence [70].

Previous genotypic analysis of cagA revealed the presence of substantial variability in the 3′ region of the gene due to the presence of repeat sequences. It has been postulated that the variability of cagA alters the immunogenicity of the protein encoded. Variations in the gene that subsequently alter its protein have been shown to correlate with clinical outcome of disease [21,71]. The presence of cagA is associated with duodenal ulcer, gastric mucosal atrophy, and gastric cancer [26,72,73]. Several studies conducted in different countries have shown that cagA positive vacA s1/m1 *H. pylori* strains were the most virulent strains and were associated with the ulcer disease [6,14,17, 21,32].

The cagA gene is possessed by approximately 68% of *H. pylori* strains, although the proportion of *H. pylori* isolates, which are cagA positive, varies from one geographic region to another [71]. Studies done in Japan have shown that more than 90% of *H. pylori* strains are cagA positive [74], while in the United States [104] and Europe these percentages are much lower [75]. In Europe it was reported to be present in 60-70% of European isolates [46] and approximately 50-60% of the *H. pylori* strains contain this gene [76].

Recent studies have provided a molecular basis for the pathological actions of CagA on gastric epithelial cells. Following the attachment of cagA-positive *H. pylori* strains to gastric epithelial cells, CagA is directly injected from the bacteria into the cells via the bacterial type IV secretion system and undergoes tyrosine phosphorylation in the host cells [69,77,78,79,80]. Tyrosine phosphorylation of CagA occurs at the unique Glu–Pro–Ile–Tyr–Ala (EPIYA) motifs present in the C-terminal region [81,82,83]. It has been suggested that *H. pylori* does not arbitrarily inject CagA into any mammalian cell line. On the contrary, there is apparently some degree of cell type specificity involved in the process of type IV secretion [105]. Furthermore, it was recently confirmed that translocated CagA forms a physical complex with Src homology 2 domain-containing protein-tyrosine phosphatase (SHP-2) in a

phosphorylation-dependent manner and deregulates its enzymatic activity [83]. SHP-2 is known to play an important positive role in mitogenic signal transduction [84]. Furthermore, SHP-2 is actively involved in the regulation of spreading, migration, and adhesion of cells [85,86]. In addition to SHP-2, CagA has been reported to bind Grb2, c-Met receptor, or phospholipase Cγ (PLCγ) in a manner independent of CagA tyrosine phosphorylation [110]. Collectively, these findings indicate that CagA functions as a bacterial adaptor/scaffolding protein that recruits multiple cellular proteins and deregulates a variety of cellular functions [111].

H. pylori can be divided into distinct populations with different geographical distributions [42,87]. It has been reported that large sequence differences distinguish the cagA gene fragments from Asian strains and other strains [88,89]. Recently, it was also demonstrated that the predominant CagA protein isolated in East Asia, where gastric cancer is prevalent, has a distinct sequence at the region that corresponds to the repeat sequence of Western CagA [89]. The phosphorylation sites are located in the repeat region of CagA [83]. After tyrosine phosphorylation, this East Asian-specific sequence confers stronger SHP-2 binding and transforming activities than the Western-specific sequence [89].

A 98% association of cagA positive strains with peptic ulcer disease was reported in Taiwanese patients suggesting that cagA positive phenotype cannot be used as a single marker in high-risk patients in Taiwan, although several reports associate the detection of cagA with PUD. The high prevalance of cagA positive isolates in Taiwan and their genetic divergence from other published strains was speculated to be specific populations of *H. pylori* strains within Taiwanese individuals [90].

2.2.3 Vacuolating Cytotoxin Gene A (vacA)

The VacA toxin produced by approximately 50 % of *H. pylori* strains is an important virulence factor that induces formation of vacuoles in mammalian cells in vitro and leads to cell death [23]. The toxin is encoded by vacA gene which is virtually present in all *H. pylori* strains [24].

It has been reported that DNA sequence analysis of *H. pylori* virulence genes revealed a complex pattern of point mutations, deletions and insertions in the vacA gene [25]. The vacA gene contains two variable regions. The signal s-region exists as s1 or s2

allelic types. Among type s1 strains, subtypes s1a, s1b, and s1c have been identified. The middle m-region exists as the m1 or m2 allelic type [14,15,26,27]. Among type m1, three subtypes have been identified, designated m1a, m1b, and m1c [28,29]. Production of the vacuolating cytotoxin is related to the mosaic structure of vacA. In general, type s1/m1 and s1/m2 strains produce high and moderate levels of toxin, respectively, whereas s2/m2 strains produce little or no toxin [14]. A recent study done in Italy indicated that mixed *H. pylori* infection harbouring in one patient is significantly related to strains that are more resistant to antibiotics and with a more virulent genotype (vacA s1m1/s1m2, cagA, iceA1) than strains responsible for single infection [107]. *H. pylori* strains that possess a type s1/m1 vacA allele are associated with an increased risk of gastric cancer and enhanced gastric epithelial cell injury compared with vacA s2/m2 strains [30,31].

A recent study has demonstrated that strains with genotype vacA s1/m1, but without s2/m2 mediate channel activity and cellular vacuolation in intoxicated cells, which results in a detectable reduction of mitochondrial transmembrane potential and the release of cytochrome c. This suggests a functional correlation between VacA channel activity and changes in mitochondrion membrane permeability [27,109].

It has been indicated that a total of 10 direct repeats is found within the coding region of vacA gene. These are: 6 direct repeats of 12 bp, 3 direct repeats of 13 bp, and 1 direct repeat of 15 bp in length [32]. There is an approximately 70% nucleotide sequence similarity in vacA gene between toxin+ and toxin- strains of *H. pylori* [7].

VacA is produced as a 140 kDa precursor polypeptide, the carboxy-terminal 45 kDa of the precursor has autotransporter activity and is cleaved from the protein after translocation across the outer-membrane [8,33]. The toxin is released from the bacteria as a high molecular mass oligomeric protein consisting of several copies of an approximately 95 kDa polypeptide [7,34]. Each monomer is further structured in two distinct subunits of approximately 37 kDa and 58 kDa. The 37 kDa subunit contains the functional vacuolating activity [35], and the 58 kDa subunit is responsible for receptor binding and interaction with the target cells [36]. It has been reported that some cell components like endocytic vesicles, mitochondria, the cytoskeleton, and the epithelial cell-cell junctions are targetted by VacA [37].

Some studies indicated that there are two forms of VacA, called m1 and m2 [7,14], which differ in their capacity to bind target cells [38]. The m2 form fails to bind and hence to intoxicate HeLa cells, but is fully active if expressed intracellularly by DNA transfection. Both forms bind and intoxicate a rabbit kidney cell line RK13 and primary cells from human gastric biopsies. The region of the protein, which defines target cell specificity is within a region of about 300 amino acids in the 58 kDa subunit which differs by about 50% in amino acid sequence between the two forms of the protein [39]. VacA protein is expressed by 66% of DU and by 65% of GU patients whereas it is expressed by 38% of nonulcer dyspepsia (NUD) patients [40].

2.3 THE ASSOCIATION BETWEEN *H. PYLORI* AND GASTRIC CANCER

Clinical studies have revealed that eradication of *H. pylori* leads to a reduction of the risk for gastric cancer recurrence and to the regression of MALT lymphoma [44,91,92]. Although *H. pylori* is clearly a gastric pathogen, the means by which it causes distinct diseases remain unclear. It is unlikely that *H pylori* infection alone is responsible for the development of gastric cancer. Rather, *H pylori* may produce an environment conducive to carcinogenesis and interact with other lifestyle and environmental exposures [108]. It has been proposed that bacterial, host genetic, and environmental factors interact to cause gastric diseases [31,93].

It has been indicated that the relative risk for development of gastric cancer was 3.8 fold higher in patients with *H. pylori* infection than those without [94]. Many studies on experimental animals supported the fact that, Mongolian gerbils infected with *H. pylori* showed that 37% of the animals developed adenocarcinoma, compared to none in the controls [95].

The association of cagA positive strains with gastric carcinoma has been evaluated by the detection of the cagA gene strains isolated from gastric carcinoma patients or by the detection of specific against CagA in sera of patients collected years before the diagnosis or when the disease is established. In a study in which the presence of cagA gene was directly evaluated, a high prevalance of cagA positive strains was observed in

gastric carcinoma, but no difference was observed when these patients were compared with those with peptic ulcer or gastritis [96].

CHAPTER 3

MATERIALS AND METHODS

3.1 COLLECTION OF BIOPSY SAMPLES

H. pylori isolates from 52 patients (32.6% females) of 28-97 years of age (average 52) attended the İstanbul Eğitim Hospital were studied. Three biopsies taken from antral part of the stomach of patients with duodenal ulcer, gastric ulcer and gastritis were analyzed. One biopsy was used for CLO test and the others for multiplex PCR assay.

3.2 DNA EXTRACTION

The method described by Chattopadhyay et al. [113] for DNA extraction by boiling biopsies in water was used. The QIAamp DNA Mini Kit (Qiagen Co., Hilden, Germany) was also used for comparison between the two methods.

The QIAamp DNA Mini Kit Contents (QIAamp DNA Mini Kit 250, Catalog No: 51306):

Number of preparations: 250
QIAamp Spin Columns: 250
Collection Tubes (2ml): 750
Buffer AL: 54 ml
Buffer ATL: 50 ml
Buffer AW1: 95 ml
Buffer AW2: 66 ml

Buffer AE: 110 ml

Proteinase K: 6 ml

In order to isolate DNA from biopsy samples the following procedure was performed according to the instruction manual of QIAamp Mini Kit.

1. Biopsy sample (of 25 mg or less) was placed in a 1.5 ml microcentrifuge tube and 180 µl Buffer ATL was added (The volume of Buffer ATL should be increased proportionally if the weight of the biopsy sample is larger than 25 mg).

2. 20 µl Proteinase K was added, mixed by vortexing and incubated at 56°C overnight in a shaking water bath until the tissue was completely lysed (The volume of proteinase K should be increased proportionally if the weight of the biopsy sample is larger than 25 mg). Lysis time varies varies depending on the type of tissue processed. Lysis is usually complete in 1-3 h and lysis overnight is possible.

3. The 1.5 ml microcentrifuge tube was centrifuged briefly to remove drops from inside of the lid.

4. 200 µl Buffer AL was added to the sample, mixed by pulse-vortexing for 15 s, and incubated at 70°C for 10 min. The 1.5 ml microcentrifuge tube was centrifuged briefly to remove drops from inside the lid.

5. A 200 µl ethanol (96-100%) was added to the sample, and mixed by pulse-vortexing for 15 s. After mixing, 1.5 ml microcentrifuge tube was centrifuged briefly to remove drops from inside the lid.

6. The all mixtures from step 5 were carefully applied to the QIAamp Spin Column (in a 2 ml collection tube) withouth wetting the rim. The cap was closed, and centrifuged at 8000 rpm for 1 min. Then, the QIAamp Spin Column was placed in a clean 2 ml collection tube (provided), and the collection tube containing the filtrate was discarded.

7. The QIAamp Spin Column was carefully opened and 500 µl Buffer AW1 was added without wetting the rim. The cap was closed, and centrifuged at 8000 rpm for 1 min.

The QIAamp Spin Column was placed in a clean 2 ml collection tube (provided), and the collection tube containing the filtrate was discarded.

8. The QIAamp Spin Column was carefully opened and 500 µl Buffer AW2 was added without wetting the rim. The cap was closed and centrfuged at 14000 rpm for 3 min.

9. The QIAamp Spin Column was placed in a clean 1.5 ml microcentrfuge tube (not provided), and the collection tube containing the filtrate was discarded. The QIAamp Spin Column was carefully opened and 200 µl Buffer AE was added. Then it was incubated at room temperature for 5 min and centrifuged at 8000 rpm for 1 min.

10. The step 9 was repeated 2 times more.

11. DNA was stored at -20°C.

3.3 MULTIPLEX PCR AND PRIMERS

The method described previously [113] was used with some modifications. Primer concentrations and annealing temperature adjustment were applied. The primers used for the amplification of vacA alleles and cagA genes were listed in table 3.1. In addition, the Chi-square test with Yates correction was used for statistical analysis. Significance was defined as *P* value of <0.05.

Table 3.1 Primers used for the amplification of vacA alleles and cagA

Genotype	Primer	Primer sequence	Size (bp)	Reference
vacA s1s2	VAI-F VAI-R	5'-ATGGAAATACAACAAACACAC-3' 5'-CTGCTTGAATGCGCCAAAC-3'	259/286	14
vacA m1m2	VAG-F VAG-R	5'-CAATCTGTCCAATCAAGCGAG-3' 5'-GCGTCAAAATAATTCCAAGG-3'	567/642	112
cagA	cag5c-F cag3c-R	5'-GTTGATAACGCTGTCGCTTC-3' 5'-GGGTTGTATGATATTTTCCATAA-3'	350	113

Reagents and Buffers:

a. Primers: Lyofilized (Bio Basic Inc., Ontario, Canada)

b. Pure Water: RNAse free (Bio Basic Inc., Ontario, Canada)

c. dNTP mixture: 2.5 mM each (Takara Bio Inc., Shiga, Japan)

d. $MgCl_2$: 25 mM (Takara Bio Inc., Shiga, Japan)

e. Ex Taq DNA Polymerase: 5 units/µl (Takara Bio Inc., Shiga, Japan)

f. Ex Taq Buffer: 10X, Mg^{2+} free (Takara Bio Inc., Shiga, Japan)

3.3.1 Calculations of the Reaction Mixture

The reaction mixture for multiplex PCR was prepared according to Chattopadhyay et al. [113]. Multiplex PCR was performed in a 25 µl reaction mixture containing 10 µl DNA, 25 pmol of each primers, 0.25 mM of each dNTP, 0.9 U of Ex Taq DNA polymerase, 1.5 mM of $MgCl_2$, 10X Ex Taq buffer, and pure water.

Primer Stock Preparation:

Primer stock solutions were prepared according to the instruction manual supplied by the manufacturer (Bio Basic Inc., Ontario, Canada).

1. VAI-F: To prepare 100 µM stock solution; 85 µl pure water was added to the lyofilized primer.

2. VAI-R: To prepare 100 µM stock solution; 110 µl pure water was added to the lyofilized primer.

3. VAG-F: To prepare 100 µM stock solution; 95 µl pure water was added to the lyofilized primer.

4. VAG-R: To prepare 100 µM stock solution; 95 µl pure water was added to the lyofilized primer.

5. cag5c-F: To prepare 100 µM stock solution; 110 µl pure water was added to the lyofilized primer.

6. cag3c-R: To prepare 100 µM stock solution; 88 µl pure water was added to the lyofilized primer.

Primer Calculation:

To obtain 25 pmol of each primers the following formula was used: (n = M x V) were n: the desired amount of primer to be placed in the reaction mixture in moles, M: molarity of primer stockand and V: the desired volume.

1. VAI-F: (Original Stock = 100 µM)

   n = M x V

   25 pmol = 100 µM x V

   V = 0.25 µl.

2. VAI-R: (Original Stock = 100 µM)

   25 pmol = 100 µM x V

   V = 0.25 µl.

3. VAG-F: (Original Stock = 100 µM)

   25 pmol = 100 µM x V

   V = 0.25 µl.

4. VAG-R: (Original Stock = 100 µM)

   25 pmol = 100 µM x V

   V = 0.25 µl.

5. cag5c-F: (Original Stock = 100 µM)

   25 pmol = 100 µM x V

   V = 0.25 µl.

6. cag3c-R: (Original Stock = 100 µM)

25 pmol = 100 µM x V

V = 0.25 µl.

dNTP Calculation:

To calculate the amount of dNTP to be placed in the reaction mixture the following formula was used: (M1 x V1 = M2 x V2)

2.5 mM x V1 = 0.25 x 25 µl

V1 = 2.5 µl.

$MgCl_2$ Calculation:

To calculate the amount of $MgCl_2$ to be placed in the reaction mixture the following formula was used: (M1 x V1 = M2 x V2)

25 mM x V1 = 1.5 mM x 25 µl

V1 = 1.5 µl.

Ex Taq DNA Polymerase Calculation:

To calculate the amount of Ex Taq DNA Polymerase to be placed in the reaction mixture the following proportion was used.

Original Stock: 5 units/ µl

1 µl 5U
x µl 0.9U
x = 0.18 µl.

Ex Taq Buffer Calculation:

To calculate the amount of Ex Taq DNA buffer to be placed in the reaction mixture the following formula was used: (M1 x V1 = M2 x V2)

10X x V1 = 1X x 25 µl

V1 = 2.5 µl.

Pure Water Calculation:

DNA: 10 µl

Ex Taq DNA Polymerase: 0.18 µl

Ex Taq Buffer: 2.5 µl

$MgCl_2$: 1.5 µl

dNTP: 2.5 µl

Primers: 0.25 µl (each)

Total volume of the reaction mixture is 25 µl.

Therefore: 10 + 0.18 + 2.5 + 1.5 + 2.5 + (0.25 x 6) = 18.18, 25 -18.18 = 6.82 µl pure water.

3.3.2 DNA Amplification

The thermal cycler (Techne, Cambridge, UK) was used for multiplex PCR assay. The products were amplified under the following conditions: 3 min at 94°C for initial denaturation followed by 35 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C, with a final round of 10 min at 72°C [113].

3.4 AGAROSE GEL ELECTROPHORESIS

Preparation of the gel:

A 1% agarose gel was used to detect PCR products.

1. 0.5 g of agarose (Sigma, St. Louis, USA) was mixed with 50 ml of 0.5X Tris-borate EDTA (TBE) buffer.

2. Then it was heated until boiling.

3. The gel was cooled to 40°C and 3.5 µl Ethidium bromide was added. The gel was then poured and a comb was placed in the gel.

Loading:

1. 10 µl PCR product was mixed with 2 µl bromophenol blue as a tracking dye.

2. 10 µl PCR product was then put in each slot.

3. 1 µl of a 100 bp DNA Ladder (MBI Fermentas, Hanover, MD, USA) was mixed with 1 µl deionized water and 1 µl bromophenol blue. Then 5 µl of this mix was put into the side slot as a molecular marker.

4. The gel was run at 95 V in 0.5X TBE buffer for 50 min.

5. The gel was placed in Gel Doc 2000 (Biorad, Milan, Italy) apparatus and the bands were detected under UV transilluminator.

CHAPTER 4

RESULTS

The presence of *H. pylori* in the gastric biopsies was detected by CLO test and PCR. *H. pylori* was positive in 44 (84.6%) of 52 of the patients of which 30 (85.7%) of 35 patients with DU, 7 (100%) of 7 with GU and 7 (70%) of 10 patients with gastritis. DNA isolation by the boiling method revealed no PCR amplicons, thus the entire DNA isolation was carried out using the QIAamp Kit. The cagA gene was detected in 26 (86.6%) of 30 patients with DU, 5 (71.4%) of 7 with GU and 4 (57.0%) of 7 patients with gastritis The differences among the PUD and gastritis patients were statistically significant (P <001). The distribution of the vacA alleles among the cagA-positive patients was as follows: in DU patients (n=26), 21 (80.7%) had vacA s1m1, 4 (15.3%) s1m2, and 1 (3.8%) s2m2 genotype, in GU patients (n=5), 3 (60.0%) revealed s1m1, and 2 (40.0%) s1m2 genotype while the gastritis patients (n=10), showed 3 (75.0%) s1m2 and 1 (25.0%) s2m2 genotype (Table 4.1). The distribution of the vacA alleles among the cagA-negative patients showed that DU patients (n=4) had s1m2 genotype, GU patients (n=2) had s1m2 genotype and the gastritis patients (n=3) showed 2 with s1m2 and 1 with s2m2 genotype (Table 4.2). We have confirmed these results by using single primer pairs for PCR. We have not encountered any changes in the amplicon sizes by this technique and the intensity of the amplicon bands was not uniform as indicated previously. In addition we have detected a band of approximately 420bp below the m1 band location in isolates from two patients with DU and one with GU. Table 4.3 shows the deletions detected in m1 region of vacA gene. The ages of the patients were ranging from 28-97. The mean age of patients was 52. Among 35 patients with DU, 24 (68.5%) were male and 11 (31.4%) were female. 5 (71.4%) patients with

GU were male and 2 (28.5%) were female. 6 patients (60%) with gastritis were male and 4 (40%) were female (Table 4.4).

In Figure 4.1, lane 2, 3, 4, 5 were of DU patients and lane 1 was GU patient. Lane 1 revealed the cagA, vacA s1m1 genotype and a 140 bp deletion was detected in m1. Lane 2 revealed the cagA, vacA s1m1 genotype. In lane 3, cagA, vacA s1m1 genotype and a 140 bp deletion in m1 were detected. Lane 4 revealed the cagA, vacA s2m2 genotype. Lane 5 revealed cagA, vacA s1m1 genotype and a 140 bp deletion was detected in m1.

Figure 4.2 shows the gel pattern of multiplex PCR assay for 3 patients with DU. The lane M represents the 100 bp marker (Fermentas). In lane 1 cagA, vacA s1m1 and in lane 2 cagA, vacA s1m2 genotypes were detected.

In Figure 4.3, all lanes were DU patients. In lane 3 and 4, cagA, vacA s1m1 genotype in lane 5, cagA, vacA s1m2 genotypes were detected.

Figure 4.4, 4.5 and 4.6 show the spectrophotometer analysis of extracted DNA samples from patients with DU, GU and gastritis respectively. Peak at 260 nm indicates the presence of extracted DNA.

Table 4.1 *H. pylori* cagA-positive vacA genotypes and the distribution percentages among peptic ulcer disease patients

No. of Patients	*H. pylori* (+)	cagA+	vacA s1/m1	vacA s1/m2	vacA s2/m2	vacA s2/m1
DU = 35	30 (85.7)	26 (86.6)	21 (80.7)	4 (15.3)	1 (3.8)	0
GU = 7	7 (100)	5 (71.4)	3 (60.0)	2 (40.0)	0	0
G = 10	7 (70)	4 (57)	0	3 (75)	1 (25.0)	0
Total = 52	44 (84.6)	35 (79.5)	24 (68.5)	9 (25.7)	2 (5.7)	0

DU: Duodenal ulcer, GU: Gastric ulcer, G: Gastritis

Table 4.2 *H. pylori* cagA-negative vacA genotypes and the distribution percentages among peptic ulcer disease patients

No. of Patients	*H. pylori* (+)	cagA-	vacA s1/m1	vacA s1/m2	vacA s2/m2	vacA s2/m1
DU = 35	30 (85.7)	4 (13.3)	0	4 (100)	0	0
GU = 7	7 (100)	2 (28.5)	0	2 (100)	0	0
G = 10	7 (70)	3 (42.8)	0	2 (66.6)	1 (33.3)	0
Total = 52	44 (84.6)	9 (20.4)	0	8 (88.8)	1 (11.1)	0

DU: Duodenal ulcer, GU: Gastric ulcer, G: Gastritis

Table 4.3 Deletions of *H .pylori*

No. of *H. pylori* (+) patients	vacA m1 (140 bp deletion)
DU = 30	2
GU = 7	1

DU: Duodenal ulcer, GU: Gastric ulcer

Table 4.4 Gender of patients and their percentages

No. of Patients	Male	Female
DU = 35	24 (68.5)	11 (31.4)
GU = 7	5 (71.4)	2 (28.5)
G = 10	6 (60)	4 (40)
Total = 52	35 (67.3)	17 (32.6)

DU: Duodenal ulcer, GU: Gastric ulcer, G: Gastritis

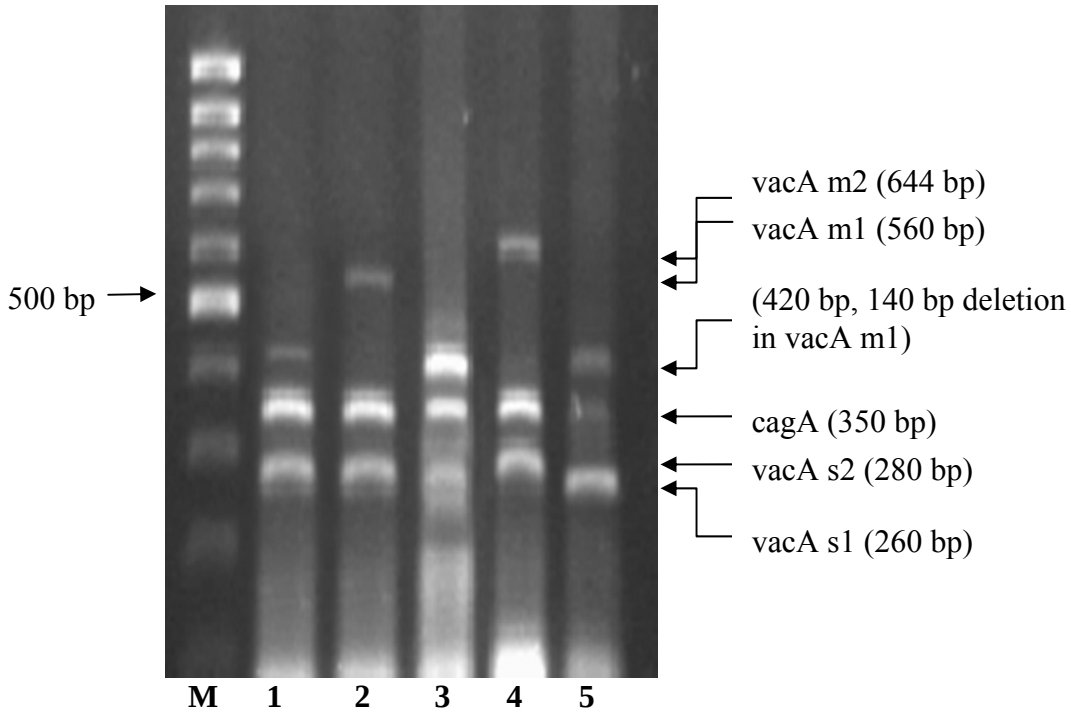

Figure 4.1 Gel electrophoresis pattern of *H. pylori* genotypes amplified by multiplex PCR. (M: molecular marker (100bp ladder), Lane 1: gastric ulcer patient, Lane 2, 3, 4 and 5: duodenal ulcer patients)

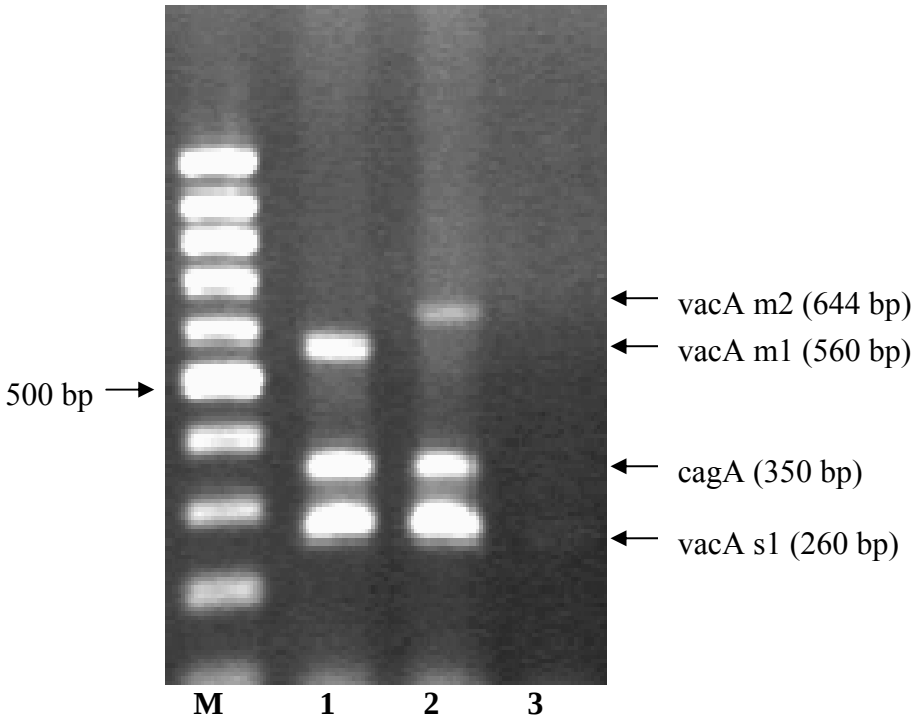

Figure 4.2 Gel electrophoresis pattern of *H. pylori* genotypes amplified by multiplex PCR (M: molecular marker (100bp ladder), Lane 1, 2, 3: duodenal ulcer patients)

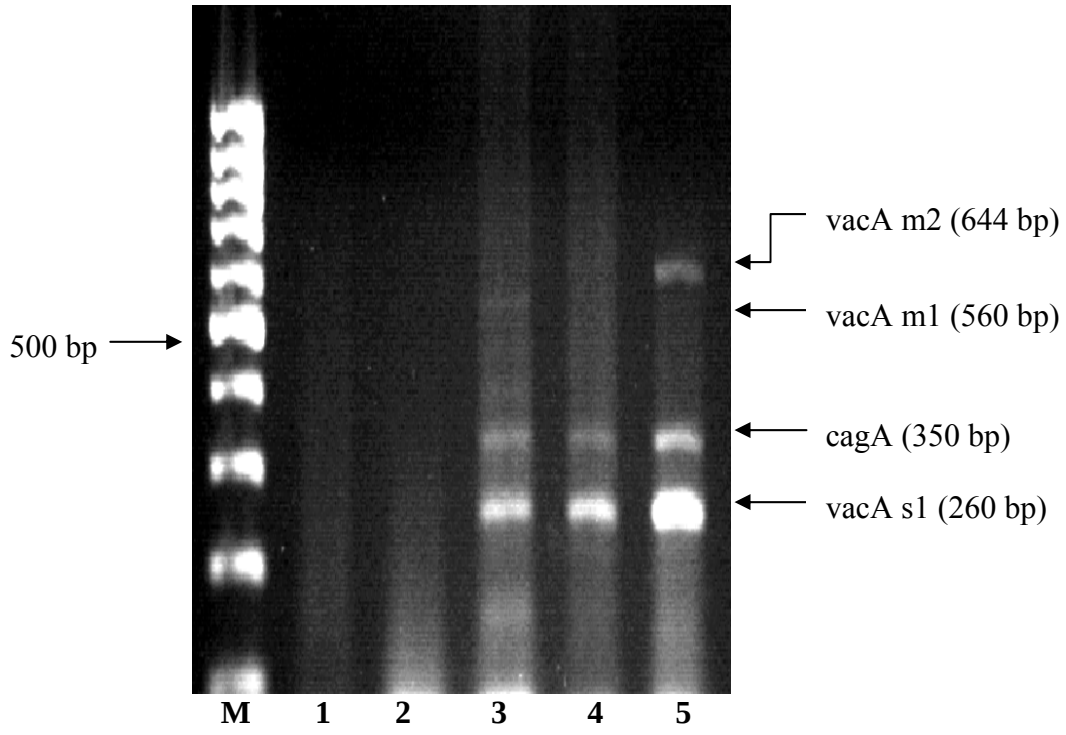

Figure 4.3 Gel electrophoresis pattern of *H. pylori* genotypes amplified by multiplex PCR (M: molecular marker (100bp ladder), Lane 1, 2, 3, 4, 5: duodenal ulcer patients)

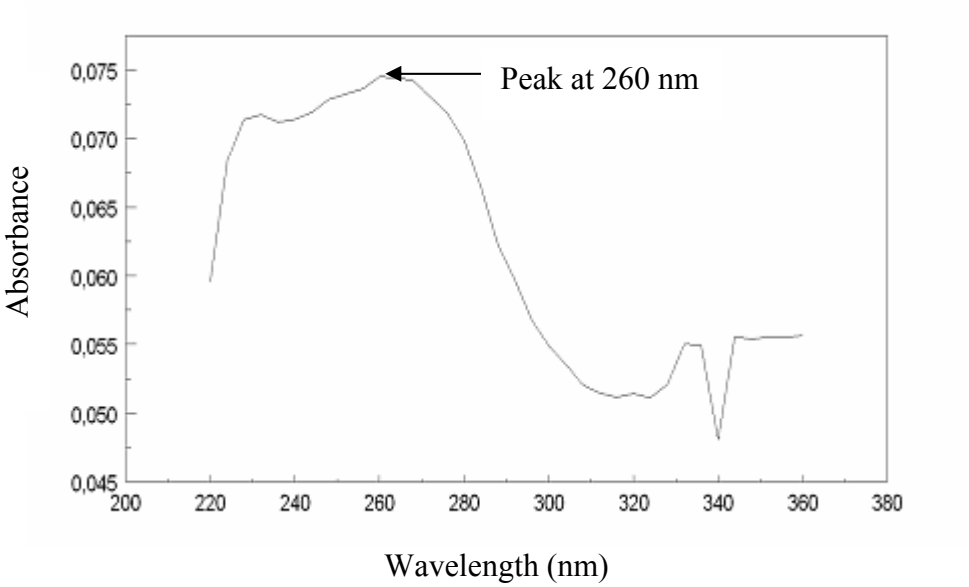

Figure 4.4 Spectrophotometer Analysis of Extracted DNA Sample From a Patient With DU (Peak at 260 nm indicates the presence of extracted DNA)

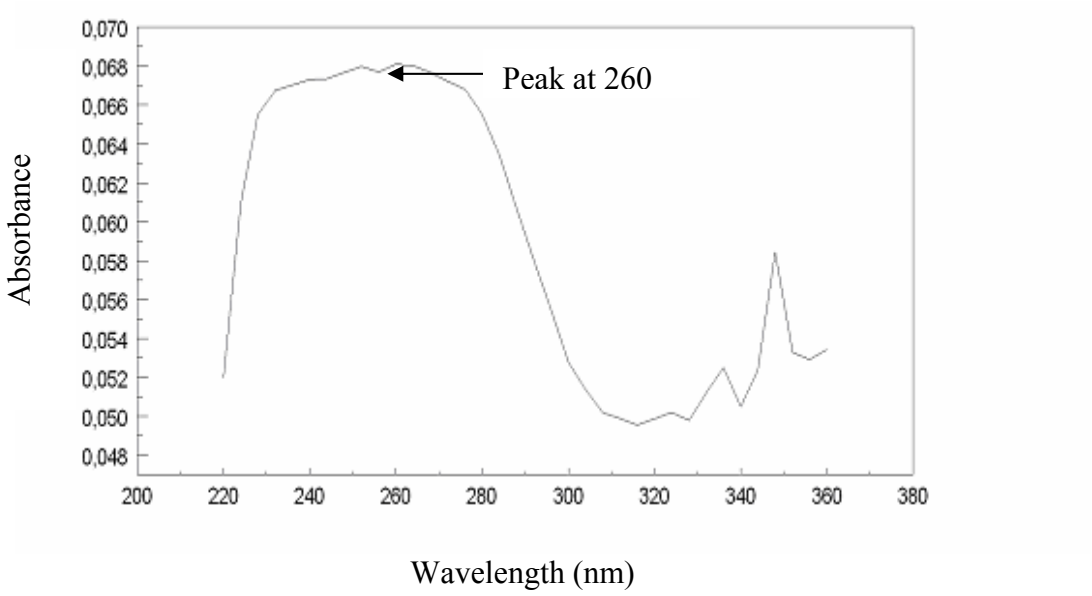

Figure 4.5 Spectrophotometer Analysis of Extracted DNA Sample From a Patient With GU (Peak at 260 nm indicates the presence of extracted DNA)

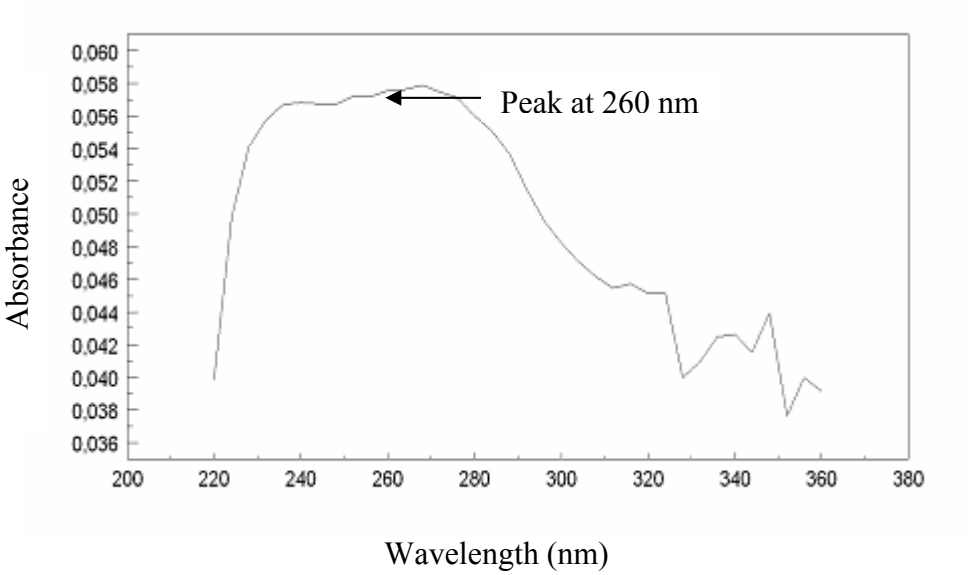

Figure 4.6 Spectrophotometer Analysis of Extracted DNA Sample From a Patient With Gastritis (Peak at 260 nm indicates the presence of extracted DNA)

CHAPTER 5

DISCUSSION

The outcome of PCR amplification of *H. pylori* DNA extracted from biopsies usually show differences in the intensity of the bands obtained since *H. pylori* is often assume patchy colonization in the gastric mucosa rather than being uniform. In amplifying such DNA using single primer pairs one can optimize the assay to obtain a uniform intensity of the amplicons however this is not always the case when performing multiplex PCR using several primer pairs. Also we were not able to amplify any of the genes from DNA extracted by boiling biopsies as reported previously [113], possibly due to the impurities found in the extracted DNA.

A key feature of *H. pylori* is the enormous genomic diversity among strains distributed worldwide. At the same time, different populations seem to be characterized by a particular geographic pattern of different *H. pylori* genotypes [20,21]. Earlier studies indicate that the distribution of cagA and vacA genes among *H. pylori* strains and their association with the severity of the disease varies among different populations [115,116,117]. In Western populations, duodenal ulceration, gastric atrophy, intestinal metaplasia and gastric carcinoma are more common among patients infected with cagA-positive than cagA-negative strains [16,20]. Yet in East Asian countries, for example China and Japan, the association between cagA positivity and severe pathology was equally frequent among both dyspeptic and non-dyspeptic patients [20]. The cagA gene

is present in 50–70% of *H. pylori* isolates in Western populations whereas it is present in >90% of isolates from Eastern populations [43,74,75,108]. In our study, we found that the majority of DU and GU but not gastritis patients (statistically significant difference) were infected with cagA-positive strains.

The vacA gene contains two variable regions, the signal (s) region that exists as s1 or s2 allelic types and the middle (m) region exists as m1 or m2 allelic types [14,15,26,27]. In general, vacA type s1m1 and s1m2 strains produce high and moderate levels of toxin, respectively, whereas s2/m2 strains produce little or no toxin [14]. It was also shown that in both East Asian and Western countries, distinct variants of *H. pylori* strains were associated with particular vacA subtypes [22]. Rudi et.al. [119], found that 94% of patients with PUD who are cagA-positive possess vacA s1m1 and s1m2 while s2m2 is only present in 6.2% of the patients and in 24.7% of gastritis patients who were cagA-negative. Chisholm et al. [101] have only looked at the vacA allelic types and found that types s1m1, s1m2 and s2m2 were present in 51.9%, 31.2% and 16.9% of strains from biopsies, respectively. In this study, we found that the majority of cagA-positive strains were associated with vacA s1m1 (68.7%) and s1m2 (25.7%). Such distribution was found more pronounced in PUD patients than gastritis, which further emphasizes the role of these virulence genes in the severity of the disease.

The vacA gene varies most markedly in its mid region, which encodes the toxin-cell binding domain. VacA type m1 binds more extensively to cells and is more closely associated with disease than is type m2 [38]. vacA also varies in its signal region, encoding the signal peptide and the N terminus of the mature toxin of type s1 is toxic, while type s2 has a short N-terminal extension on the mature toxin which abolish its

vacuolating activity [14,118]. DNA sequence analysis revealed a complex pattern of point mutations, deletions and insertions in these genes [25]. Such indicated that mutations occurring in the primer binding sites might explain some of the discrepancies previously observed in the expression of these genes [5]. Chattopadhyay et al. [113] reported for the first time a deletion of a 120 bp in the vacA m1 allele in only one strain and that there was no size difference among the amplicons examined however, Monstein et al. [25] showed wide size heterogeneity along the vacA pattern in their report. In the present study the detection of deleted band below the vacA m1 band that was similar in location to the band detected earlier [113] might suggest that such deletion most probably took place in the site coding for toxin-cell binding domain but not vacuolation-inducing domain since it appeared only in patients with more severe diseases. Such an interpretation was made based on the study reported by Ye and Blanke [120] who have analyzed the domain structure of mature VacA that is composed of a discrete amino-terminal domain (p37) and a carboxyl-terminal domain (p58). They indicated that the amino acid residues 1-422 are the minimal VacA fragment required to induce cellular vacuolation while the central region of p58 is receptor-binding domain. Further DNA sequence analysis will confirm the structure of the deleted domain of vacA m1.

CHAPTER 6

CONCLUSIONS

The multiplex PCR although rapid and an effective tool for detecting several genes in a single step system one has to adjust for optimization of the technique when genotyping *H. pylori* direct from biopsies. Starting from DNA extraction, the outcome of the results can vary depending on the method used and the distribution of *H. pylori* in the biopsy tissue. The technique allows the detection of deletions even if it is of a small magnitude. A significant association was found between the cagA-positive vacA-s1m1 genotype and PUD.

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