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Genomic Characterisation and Comparative Proteome Analysis of *Helicobacter pylori* related Gastroduodenal Pathologies

A Thesis submitted for the degree of Doctor of Philosophy (Ph. D.)

By

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April 2009
DECLARATION

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Abdurrazag Taher Nami
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SUMMARY

The Gram-negative bacterium *Helicobacter pylori* is a gastric pathogen that chronically infects more than half the world’s population. *H. pylori* infection induces various upper gastro-duodenal diseases, and there is a marked difference in clinical outcomes due to bacterial infection among different regions in the world. *H. pylori* infection causes many perturbations of gastric structure and function. The nature and extent of the perturbation relates in part to the virulence of the organism, to the host’s genetically determined response to the presence of the infection, and environmental factors that might up or down regulate the interactions. This study aimed to recruit a patient population including patients with intestinal metaplasia as well as peptic ulcer and chronic gastritis patients to find genetic markers of *H. pylori* related gastro-duodenal diseases.

Although the presence of the DNA *H. pylori* in small quantity in paraffin-embedded gastric biopsies, using the technique of nested PCR, permitted high typability of *H. pylori* genotyping of cagA and iceA genes, more individuals were found to be infected with specific genotypes in the gastric mucosa. The virulence subtype composition vacAs1/cagA+/iceA1 occurred mainly in intestinal metaplasia, whereas the vacAs2/cagA/iceA2 was found in chronic gastritis patients. Eventually, patients infected with such strains could be selected for anti-helicobacter treatment to prevent gastro-duodenal diseases later in life.

In our study, finding of multiple cagPAI genotypes was more prevalent in patients with chronic gastritis than in those with intestinal metaplasia or peptic ulcer. The presence of such strains colonizing such patients should be considered when planning therapeutic strategies, as well as the young age group of individuals whose parents have gastric cancer.
This study reports that *H. pylori* highly virulent type 1 (*cagA/vacA* positive) and triple-positive strains (*cagA/vacAslm2/babA2*) are associated with increased risk of peptic ulcer and the development of Intestinal metaplasia.

The results presented here demonstrated that *H. pylori* exhibits a high genetic diversity, such as strain specific genes which include the presence of *jhp0947* gene in certain *H. pylori* isolates, instability of the *cag* pathogenicity island (deletion of *cagA, cagE* or *cagT*), and the chromosomal rearrangements which include the locus of plasticity region which randomly in the clinical isolates.

This study found that in-frame *oipA* gene may be linked to gastro duodenal diseases because of its correlation with other virulence factors. The *oipA*"on" status was associated with *vacAslm1* and *babA2* genotypes in the presence or absence of *cagA* gene. Further investigations of such association and their implications in adherence and colonization will be necessary to confirm the role of *oipA* in *H. pylori* infection.

Although the studies on the plasticity region are preliminary, based on our findings and those of others, it is unlikely that *dupA* is itself a determinant of a specific clinical outcome in our population.

Our results shows that *hrgA* gene was independent of the clinical outcome, and had no relationship between the *cagA, vacA*, and *babA2* genotypes and the prevalence of the *hrgA* gene among *H. pylori* isolates was found, and it can not be considered as a genetic marker of *H. pylori* -related gastrodoudenal diseases. However, the plasticity region gene termed *jhp047* can serve as a marker of clinical outcomes in our population, and could be considered as a novel virulence factor of virulent *H. pylori* -related gastro-duodenal pathologies.

The results of this study showed that the proteome analysis employs solubilisation of bacterial protein extracts and their separation by two-dimensional (2D) electrophoresis, followed by western blotting could provide the basis of a non-invasive serology tool able to assess previous infection with *H. pylori*.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>2-D</td>
<td>Two-dimensional</td>
</tr>
<tr>
<td>AG</td>
<td>Atrophic gastritis</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>babA</td>
<td>Blood group antigen binding adhesin</td>
</tr>
<tr>
<td>BHI</td>
<td>Brain Heart Infusion</td>
</tr>
<tr>
<td>cagA</td>
<td>Cytotoxin-associated gene A</td>
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<td>CagA</td>
<td>Cytotoxin-associated protein</td>
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<td>cagPAI</td>
<td>Cag Pathogenicity Island</td>
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<td>CG</td>
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<tr>
<td>CLO</td>
<td>Campylobacter-like organisms</td>
</tr>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>GC</td>
<td>Gastric cancer</td>
</tr>
<tr>
<td>GU</td>
<td>Gastric ulcer</td>
</tr>
<tr>
<td>H. PYLORI</td>
<td>Helicobacter pylori</td>
</tr>
<tr>
<td>iceA</td>
<td>Induced by contact with epithelium</td>
</tr>
<tr>
<td>IARC</td>
<td>International Agency for Research on Cancer</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IM</td>
<td>Intestinal metaplasia</td>
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<tr>
<td>Kb</td>
<td>Kilobase</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo Daltons</td>
</tr>
<tr>
<td>LPS</td>
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</tr>
<tr>
<td>MALT</td>
<td>Mucosa-associated lymphoid tissue</td>
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<tr>
<td>μ</td>
<td>Micro</td>
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<tr>
<td>μg</td>
<td>Microgram</td>
</tr>
<tr>
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<td>Microlitre</td>
</tr>
<tr>
<td>NAP</td>
<td>Neutrophil activating protein</td>
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<tr>
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<td>Description</td>
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<td>-------------</td>
</tr>
<tr>
<td>Ng</td>
<td>nanogram</td>
</tr>
<tr>
<td>NUD</td>
<td>Non-ulcer dyspepsia</td>
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<td>Minute</td>
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<td>Pathogenicity island</td>
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<td>sabA</td>
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<td>SDS</td>
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<td>TBE</td>
<td>Tris borate EDTA buffer</td>
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<tr>
<td>vacA</td>
<td>Vacuolating cytotoxin A</td>
</tr>
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<td>VacA</td>
<td>Vacuolating cytotoxin protein</td>
</tr>
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<td>WHO</td>
<td>World Health Organization</td>
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DEDICATION

To my family
"you've got to be careful if you don't know where you're going because you might not get there"

Yogi Berra

IN THE NAME OF ALLAH, THE MOST GRACIOUS, THE MOST MERCIFUL

Recite, in the name of your lord who created man from alaq.
Recite and your lord is the most generous.

(Quran 96:1-3)
CHAPTER 1

GENERAL INTRODUCTION
1.1 Helicobacter pylori

1.1.1 The history of Helicobacter pylori

Throughout the nineteenth and twentieth centuries numerous reports had described the presence of urease producing spiral organisms in animal and human stomachs (Buckely and O’Morain, 1998). The presence of the gastric spiral bacteria was reported in dogs (Bizzozero, 1893), cats and mice (Salomon, 1896) as early as the 1890's. The first description of spiral bacteria in the human stomach was provided by Krienitz (1906) in a study involving patients with gastric carcinoma and nine years later, similar microorganisms were reported in the stomachs of patients with gastric and duodenal ulceration (Rosenow and Sanford, 1915). The aetiopathologic role of a bacterial pathogen in peptic ulcer disease was proposed by Steer and Colin-Jones in 1975 who reported bacteria to be present in 88% of gastric biopsies from patients with gastric ulcer. A few years later in 1983 this organism was found in patients with active chronic gastritis and named as Campylobacter pyloridis in 1984 (Warren and Marshall, 1983; Marshall and Warren, 1984).

The role of Campylobacter pyloridis in causing active gastritis was successfully reported in healthy volunteers who fulfilled Koch’s postulates (Marshall et al., 1985). Self-inoculation was also reported by Morris, further confirming the pathogenic role of the Campylobacter pyloridis (Morris and Nicholson, 1987).

During the next few years, microbial characterization of the organism was performed and the pathogenic potential of this newly discovered bacterium was fully investigated (Blaser, 1987).
This bacteria was initially named as *Campylobacter pylori*, however taxonomic features of the bacterium did not match the genus Campylobacter as previously suggested (Goodwin et al., 1989) and the name was changed to *Helicobacter pylori* (*H. pylori*).

In the presence of substantial evidence associating *H. pylori* with gastric cancer, the World Health Organization classified *H. pylori* as a type I carcinogen (IARC 1994). This led to a substantial increase in the number of peer-reviewed publications on *H. pylori* from less than 200 in 1990 to approximately 1500 per year over the last few years (Kusters et al., 2006).

In the year 2005 Warren and Marshall were awarded the Nobel Prize for their important discovery of *H. pylori* and its role in gastritis and peptic ulcer disease. (Pincock, 2005). The discovery of such a relationship between the *H. pylori* and upper gastrointestinal disorders strongly stimulated further research throughout the world.

![Image](www.bio.davidson.edu)

**Figure 1.1: Invasion *H. pylori* (www.bio.davidson.edu)**
1.1.2. Microbiology

*H. pylori* belong to the epsilon subdivision of Proteobacteria, and is classified in the *Helicobacterales* family of the Campylobacterales order (Eppinger *et al.*, 2004). *Helicobacter* species can be subdivided into two major groups including the gastric and the enterohepatic (nongastric) species. Both groups demonstrate a high level of organ specificity, such that gastric helicobacters in general are unable to colonize the intestine or liver, and vice versa (Kusters, *et al.*, 2006).

*H. pylori* are spiral-shaped, Gram-negative bacteria each measuring 0.6 – 3.5 microns, with 5-7 flagella at one end. Initial isolation conditions require an enriched medium, an atmosphere with reduced oxygen, i.e., about 5% O$_2$ and 5-10% CO$_2$ and an optimum temperature of 37°C. *H. pylori* growth is slow with small (~1mm), smooth, translucent colonies appearing after 3 days, (Andersen and Wadström 2001). *H. pylori* strains exhibit a rod-like morphology when cultured on solid medium and its spiral form is rarely seen (Goodwin *et al.*, 1990). After prolonged culture of *H. pylori* coccoid structures usually predominate (Bode *et al.*, 1993) and these forms are metabolically active. However, coccoid forms cannot be cultured in vitro (Bode *et al.*, 1993). It is not yet known whether the coccoid forms adopted by *H. pylori* after prolonged culturing are viable or dead forms of the bacterium (Kusters *et al.*, 1997).

The *H. pylori* genome has an average size of 1.6 Mb (Alm *et al.*, 1999), with a G+C% composition of 34-39 mol%. The full genome sequences of two unrelated *H. pylori* strains are available (Tomb *et al.*, 1997; Alm *et al.*, 1999). Plasmids are found in approximately 40% of isolates. However, no virulence determinants encoded by them have been found (Owen, 1999). Recently a third and fourth
genomic sequence of a *H. pylori* strain isolated from atrophic gastritis and duodenal ulcer patients are also available (Oh et al., 2006; Baltrus et al., 2009).

A unique feature of *H. pylori* is its colonization of the acidic gastric environment for the lifetime of the host. It shows a strict tissue and host tropism for the gastric mucosa of humans and primates. *H. pylori* is normally found in the less acidic antrum region (lower part) of the stomach, but colonizes the corpus (mid) region during conditions of low acid secretion. *H. pylori* survives the low pH in the stomach by producing high levels of the enzyme urease, which hydrolyzes urea into ammonia (NH$_3$) and carbonic acid. Ammonia buffers the cytosolic and periplasmic pH, as well as the microenvironment immediately surrounding the bacteria. Unlike the urease of most other bacterial species, the *H. pylori* enzyme can also be associated with the bacterial surface or shed into the medium. This appears to be due to release of urease from bacterial lysis and subsequent adsorption of the protein onto the surface of living bacterial cells. The urease enzyme is an important colonization factor for *H. pylori*, produced in high quantities by all the clinical isolates and is essential for their growth *in vivo* (Montecucco and Rappuoli, 2001).

For many bacteria including *H. pylori*, flagellum-dependent movements are essential for infection. In *H. pylori* each flagellum is approximately 3 μm long and covered by a sheath, which is a membranous layer continuous with the outer membrane (Goodwin et al., 1985). The sheath has been suggested to protect the polymeric filament core from dissociation by low pH, and from being recognized by the immune system. The *H. pylori* flagella are composed of three structural units: a basal body in the cell wall which contains the components required for flagellar rotation and chemotaxis, a flagellar filament that works as a propeller, and a hook.
that connects the flagellar filament with basal body (Spohn and Scarlato, 2001).

More than 40 proteins of *H. pylori* genome have been suggested to be involved in
regulation, secretion and assembly of the flagellar structures (Tomb *et al.*, 1997).

The flagellar filaments consist mainly of two proteins: the major flagellin FlaA and
the minor flagellin FlaB (Leying *et al.*, 1992; Suerbaum *et al.*, 1993). *flaA* -
disrupted mutants are completely non-motile whereas *flaB* mutants are still motile
(Suerbaum *et al.*, 1993). Eaton and colleagues showed that full motility was

*H. pylori* colonizes deep in the mucus layer where part of the bacterial population
is attached to the gastric epithelial cells, but the great majority of bacteria are
motile and found within the viscous mucus layer (Testerman *et al.*, 2001). The
mucus is composed of highly glycosylated proteins (mucins) and being
continuously secreted by the gastric glands and the epithelial cells to form a layer
that protects the gastric epithelial cells. It also plays an important role in
maintaining a neutral pH at the epithelial cell surfaces through its ability to retain
bicarbonate. The surface of the gastric mucus layer is continuously shed into the
gastric lumen. To avoid being cleared by mucus turnover, *H. pylori* swims towards
the gastric epithelial cells, guided by a chemical gradient in the mucus layer.
Experimentally challenged Mongolian gerbils demonstrated that *H. pylori* uses
mucus pH for chemotactic orientation. Elimination of the mucus pH gradient by
simultaneous reduction of arterial pH and bicarbonate concentration causes
bacteria to lose orientation and disseminate in the mucus layer (Schreiber *et al.*, 2004).
1.1.3. Epidemiology and transmission of infection

*H. pylori* colonize all human populations worldwide. The risk of being colonized by *H. pylori* depends on geographic area, socioeconomic status and age of the host. Most *H. pylori* transmission occurs in childhood, and in some countries up to 90% of children become infected by the age of 10 years, with reports of infection as early as the first months of life (Bardhan, 1997). Maternal-to-child and sibling-sibling transmission seem most likely because longitudinal studies have shown that the risk of acquiring the infection is highly correlated to the infection status of the mother (Weyermann *et al.*, 2006) and the siblings (Kivie *et al.*, 2005) which in turn is related to overcrowding conditions at the home (McCallion *et al.*, 1996). The higher prevalence of *H. pylori* in individuals over 40 years of age is considered to be due to a birth cohort effect rather than a continuous future risk of acquiring infection i.e. the incidence of infection being higher in the past (Cover *et al.*, 2001). In developing countries the infection can be almost ubiquitous, whereas in industrialized countries *H. pylori* infects around 30 – 50% of adults. Socioeconomic status and ethnic grouping also play an important role in *H. pylori* epidemiology and there is a higher infection rate in children from poorer countries, also members of different ethnic groups have different levels of infection even within the developed countries (Frenck and Clemens, 2003). Transmission may be related to the ability of *H. pylori* to form non-culturable coccoid forms when exposed to unfavourable environmental conditions. However, controversy exists as to whether these coccoids are alive and important for transmission (O'Rourke and Bode, 2001). There is also a high probability that no significant reservoirs exist outside the human stomach, since *H. pylori* has a rather small genome which does not support all necessary metabolic pathways for a non-parasitic life-style (Alm *et al.*, 1999; Tomb *et al.*, 1997). Thus person to person contact involving ingestion of
H. pylori from saliva, vomit, feces or recently contaminated foods or beverages would be the most likely modes of transmission.

Serological and DNA fingerprinting analyses suggested that person-to-person transmission occurs mainly within families i.e. vertical transmission instead of horizontal (epidemic) transmission, which is the most common mode of transmission for infectious diseases (Kersulyte et al., 1999; Mitchell, 2001). This theory is also supported from the fact that the same strain is frequently shared between mothers and their children, but less frequently between spouses, while transmission is less common between fathers and their children (Kivi et al., 2003; Taneike et al., 2001).

The decline in H. pylori infection incidence may be related to industrialization and improvements in socioeconomic levels and explained by the frequent use of antibiotics, improvements in sanitation, and reduced crowding (Bruce and Maarooos, 2008).

1.1.4. Clinical Diagnosis of H. pylori infection

Numerous diagnostic tests are available for determining the presence of H. pylori infection. These tests are based on either an invasive endoscopic procedure, thus allowing the collection of gastric mucosal biopsy specimens, or non-endoscopic methods, e.g. serology or the urea breath test (UBT). The sensitivity of a particular test, and therefore its applicability, may vary depending on whether it is performed before treatment or 4-6 weeks after H. pylori eradication treatment. Following eradication treatment, even if the infection is still present, the bacterial density will be much lower and this will alter the sensitivity and specificity of a test.
However, no single routine test is ideal for the detection of *H. pylori* infection, and hence, a combination of the results derived from two tests with acceptable sensitivity and specificity is recommended for accurate diagnosis (WPEHPSG, 1997).

### 1.1.4.1. Endoscopic Methods

Recent progress has been made to magnify the gastric mucosal abnormalities with narrow band imaging (Kim *et al.*, 2004), endocytoscopy (Inoue *et al.*, 2004), and confocal laser endomicroscopy (Kiesslich *et al.*, 2005). This last technique enabled detection of *H. pylori* by surface microscopy imaging of living tissue during ongoing endoscopy for the first time. Using two contrast stains, topical acriflavine and intravenous fluorescein, with an endomicroscope (Pentax, Tokyo, Japan), endoscopists were able to see clusters of bacteria as well as single bacterial cells stained by acriflavine both on the surface and in the deeper layer of the gastric epithelium. This report is a real breakthrough in current diagnostic modalities (Kiesslich *et al.*, 2005). Moving bacteria were observed at 1100x magnification, giving hope for a possible direct detection during endoscopy (Kimura *et al.*, 2006). In a recent study, magnifying endoscopy was used on a group of patients to classify the gastric surface according to four patterns: flat, irregular, papillary or nonstructured, which were then compared to the updated Sydney System for histologic gastritis. Histologic gastritis was found in 91% of the biopsy sections with a nonflat type, and among them 96% were confirmed to harbour *H. pylori* infection (Kim *et al.*, 2006). However, a number of other invasive tests are employed for *H. pylori* diagnoses which include culture, the rapid urease test (RUT) and histological examination. All of these biopsy tests are subject to the limitations of sampling error resulting from the uneven distribution of *H. pylori* in
the gastric mucosa and for this reason multiple biopsies are often taken to improve detection of the \textit{H. pylori}.

1.1.4.1.1. Rapid urease test

A urease test based on an immunological detection of urease was proposed for the first time in Japan. Its sensitivity was 96\% but its specificity only 90\% (Isomoto et al., 2006). The \textit{H. pylori} urease is an important phenotypic marker for \textit{H. pylori} positivity in gastric biopsy specimens. The rapid urease test is based on the principle that urease catalyses the hydrolysis of urea to ammonia and carbonic acid with a concomitant increase in pH. Although a number of commercially available rapid urease tests are available, the CLO test is the most widely used endoscopy-based test for \textit{H. pylori} diagnosis. A gastric biopsy specimen is placed into an agar gel containing urea and a pH indicator. The change in pH due to the presence of \textit{H. pylori} is indicated by a colour change of the agar from yellow to magenta. The time taken for this colour change to occur is dependent on the number of bacteria present in the biopsy specimen. Maximal urease activity is achieved at a temperature of 43\degree C (Mobley et al., 1988) and hence, the speed of the reaction may be increased by performing the test above room temperature (Laine et al., 1996). In most cases the test will yield a positive result within a couple of hours following inoculation, although in practice these tests are read at up to 24h. This increases the sensitivity of the test but is associated with a decrease in specificity due the presence of other urease-producing bacteria and may give rise to a false-positive result. False negative results may arise in older patients (>60 years old) where the presence of gastric atrophy or intestinal metaplasia reduces the ability of \textit{H. pylori} to colonise the gastric mucosa (Abdalla et al., 1998). The sensitivity of the CLO test is
approximately 90% in untreated patients and may be improved by using two antral biopsy specimens in one CLO test slide (DeBoer, 1997) or by incubating the test at above 37°C. However, the sensitivity of the rapid urease test depends on the number of bacteria present in the biopsy sample, a high number (at least $10^3$) of organisms being required to yield a positive result (Xia et al., 1994). Therefore, this test may be inappropriate for the assessment of *H. pylori* status in post-treatment patients because of the presence of low numbers of residual bacteria when eradication has been unsuccessful (Méraud, 1996). However a recent study demonstrated that increasing the number of gastric antral biopsies from 1 to 4 significantly improves the sensitivity of the CLO test by eliminating sampling error, and hastens the time needed by the test to become positive for the diagnosis of *H. pylori* infection (Siddique et al., 2008). Recently two new rapid urease tests (RUT) based on pH change were also evaluated. Unfortunately, the dry RUT (GUT test) was not reliable at a 15-minute reading time (van Keeken et al., 2006). The motility indole urease test (MIU), in contrast, had a high sensitivity (Kumala, 2006).

### 1.14.1.2. Culture

The gold standard for diagnose of *H. pylori* infection is to culture the organism from biopsy samples obtained during endoscopy. Care must be taken to ensure that the patients did not receive antibiotics or antisecretory drugs, especially proton pump inhibitors (PPI). Although PPI have no direct antimicrobial effect at the concentration present in the gastric mucosa (Méraud et al., 1991), they however indirectly interfere with *H. pylori* distribution in the stomach by changing the pH of its bacterial niche, leading to its disappearance in the antrum. The recommendation is not to consume these drugs two weeks prior to endoscopy.
The success rate of isolation of *H. pylori* by culture is dependent on the transport condition from the endoscopy room to the microbiology laboratory and handling of the gastric biopsy specimen during processing. Gastric biopsies are transported in a number of transport media, such as nutrient Brucella broth, or Stuart's medium, and saline or in a commercially available transport medium (e.g. Portagerm, bio Merieux). It is important to maintain the specimen at 4 °C to avoid dessication and to prevent the overgrowth of accompanying bacteria. Gastric biopsies may be inoculated directly onto solid microbiological media by smearing the biopsy (mucosal side downwards) over the surface of the agar but higher isolation rates and a more homogenous growth are achieved when the biopsy specimen is homogenised with a tissue grinder (Mégraud, 1996; WPEHPSG, 1997).

Various media are available for isolation including Muller-Hinton agar, Columbia agar, Wilkins Chalgren agar, Brain Heart Infusion agar and GC* agar (Oxoid) which are supplemented with either horse, sheep or human blood at concentrations ranging from 5 to 10% (v/v), ferrous sulfate, sodium pyruvate, and swine mucin have been proposed to enhance *H. pylori* growth (Jiang and Doyle, 2000). Contamination compromises the sensitivity of the test and, therefore, selective supplements (vancomycin, nalidixic acid, trimethoprim, amphotericin B) may also be added to the agar to inhibit the growth of other bacteria and fungi. The Dent supplement, a modification of Skirrow’s formula in which cefsulodin replaces polymyxin and amphotericin B is added is commercially available (Dent and McNulty, 1988). The choice of a particular medium will depend on local facilities and preferences but a combination of a selective and a non-selective medium should be used to increase sensitivity (Tee et al., 1991; Piccolomini et al., 1997).

Plates are incubated at 37°C under microaerophilic conditions in 98% humidity
for five to seven days. Incubation may be prolonged for up to 10 days in the case of a post-treatment evaluation. The specificity of culture is the best (100%) of all of the diagnostic tests. In order to minimise the risk of sampling error, it is advisable that two antral biopsy samples are taken for culture in untreated patients and that biopsy samples from both the antrum and corpus are collected for post-treatment assessment of *H. pylori* status (WPEHPSG, 1997; Glupczynski, 1998). High sensitivity is achieved in certain centres with expertise and experience of the method (van Zwet et al., 1993; Lerang et al., 1998) but the published data suggest that the majority of investigators fail to reach high sensitivity rates in clinical practice. Microbiological culture is essential for antimicrobial susceptibility testing and provides important information for the management of *H. pylori* infection. Isolation of the organism is also required to investigate microbe-host interactions, for molecular biology techniques, e.g., the detection of the presence of certain *H. pylori* genes by the Polymerase Chain Reaction (PCR), and for the genetic comparison of various strains. Culture continues to be the only test allowing for a comprehensive analysis of pathogen characteristics and susceptibility to antibiotics (Hirschl and Makristathis, 2007).

1.1.4.1.3. Histology

As a diagnostic test, histology can estimate the extent of the *H. pylori* infection and is the only test that simultaneously determines the histopathological state of the gastric mucosa. It has been recommended that two biopsy samples should be taken from each of the antral and corpus sites of the stomach for the histological assessment of *H. pylori* infection (Price, 1991). Gastric biopsy specimens are transported in a fixative where they are processed and subsequently sectioned and stained for examination. Routine haematoxylin and
eosin stain offers the best histological assessment of gastritis but the identification of *H. pylori* may be inaccurate when there is debris on the gastric surface or when *H. pylori* infection is scanty (Price, 1996). The Giemsa stain is widely used and is an easy, rapid and effective staining method. Although these stains are not specific for *H. pylori*, the shape and the association of the bacteria with the luminal surface of the gastric epithelium allow distinction between *H. pylori* and other upper digestive tract bacteria that are occasionally present. Immunocytochemistry is available for the specific identification of *H. pylori* infection (Dunn *et al.*, 1997).

The quality of biopsy specimen will significantly affect the sensitivity and specificity of the histological test. Therefore, it is essential that adequate mucosal biopsy specimens are taken for histological assessment and that they are properly orientated and processed. Furthermore, the efficacy of the test will, to a large extent, also depend on the experience of the observer. Inter-observer variability is high, particularly when the infection is scanty. When biopsy specimens have been examined by several pathologists the concordance of results has been poor (Kolts *et al.*, 1993; El-Zimaity *et al.*, 1996; Maconi *et al.*, 1999). Histological detection can reach a sensitivity of 95% under optimal conditions, while quality of the material and the pathologist's expertise are the limiting factors. The presence of inflammation (lymphocytes) and especially inflammatory activity (polymorphs) is an obvious indication for an in-depth search for *helicobacter* organisms. The specificity is also in the range of 95%. The presence of other bacteria on the mucosa may be a cause of false-positive results if there are few bacteria with atypical morphology. However, the pathologist can use immunohistochemistry or fluorescein (fluorescence in situ hybridization [FISH]) for confirmation. The latter
method is now commercially available since it can also be used for antimicrobial susceptibility testing. (Méraud and Lehours 2007).

Histological results must be reported according to guidelines drawn up in 1990, known as the Sydney system (Price, 1991). The presence of bacteria in the corpus and in the antrum is expressed semiquantitatively on a scale of 0 to 3. In addition, the histological characteristics of the gastric mucosa (inflammation, activity, atrophy, intestinal metaplasia) are also reported. The recent update of the Sydney system proposes inclusion of biopsy specimens from the incisura, an area where premalignant lesions are commonly found (Dixon et al., 1996).

1.1.4.1.4. Molecular Methods

During recent years, molecular techniques have been widely used for the diagnosis of H. pylori infection as well as for analyses of diversity, virulence, persistence patterns of these bacteria, and in order to detect H. pylori and other Helicobacter species, determine susceptibility to antibiotics, and for typing reasons. In a recent study, a novel and quick identification system for H. pylori detection which is a combination of the endoscopic brushing technique and the loop-mediated isothermal amplification method (LAMP). LAMP assays provide clinicians with results quickly. LAMP assay is a novel gene amplification strategy in which all reactions are conducted under isothermal conditions using a single type of enzyme. This method has high amplification efficiency and provides faster amplification than the previous PCR methods (Enosawa et al., 2003; Yoshida et al., 2005). Using this new technique among the samples from 200 patients, 123 brushing samples were H. pylori positive using LAMP primers constructed for the glmM gene within a 90-minute detection time with 100% sensitivity and
specificity, whereas 100 patients were positive when only biopsy samples were tested (Minami et al., 2006).

One of the best known molecular methods being used for microbial diagnosis is the PCR. This technique is widely available for the detection of various bacteria including *H. pylori*. The reaction cycle is composed of three different temperature-dependent steps. Double-stranded target DNA is heated to 94°C to allow the complete separation of the two complementary DNA strands. The temperature is then lowered to allow annealing of two short single-stranded DNA primers (usually 10-30 bases long of opposite polarity) which form a stable DNA duplex. The specificity of the PCR is governed by the degree of homology between the primer sequence and the DNA template. Following primer annealing, the temperature is then raised to 72°C and the DNA that spans between the primers is extended by the incorporation of deoxynucleotides by a heat-stable DNA polymerase. Thereafter, the cycle of DNA denaturation, primer annealing and DNA extension is repeated resulting in the exponential amplification of the target DNA.

The DNA amplification products of the reaction are visualized by agarose gel electrophoresis. A considerable number of PCR primers have been developed (Clayton et al., 1992; Kawamata et al., 1996) and evaluated for the detection of *H. pylori* DNA in a wide range of specimens including gastric biopsy material (van Zwet et al., 1993; Lage et al., 1995; Lu et al., 1999), gastric juice aspirates (Kawamata et al., 1996), and dental plaque (Gramley et al., 1999). In theory, PCR is a rapid and accurate technique that can detect one copy of the target DNA. The nature of the technique is such that it detects *H. pylori* DNA and as a consequence, the method does not distinguish between viable and non-viable bacteria. In clinical practice, the technique has high sensitivity and false-positive results may occur in biopsy specimens that contain *H. pylori* DNA derived from endoscopes that have
not been adequately washed (Roosendaal et al., 1999). At present PCR is regarded largely as a research tool and has no routine clinical application. A recent study demonstrates that PCR testing was valuable in providing improved detection rates and antibiotic susceptibility information when *H. pylori* culture was unsuccessful (Chisholm and Owen, 2008).

1.1.4.2. Non-Endoscopic methods

1.1.4.2.1. Urea Breath Test (UBT)

The Urea Breath Test is considered to be the most accurate non-invasive method to detect *H. pylori* infection. Although the original description of the $^{13}$C-UBT was published nearly 20 years ago, and many modifications have been introduced since then, the test has not yet been definitely standardized. There are differences among protocols regarding methods of analyzing breath samples, types of test meal, doses of urea, time of breath sampling, and cut-off values (Fangrat et al., 2006). The UBT, like the rapid urease test, depends on the ability of the *H. pylori* urease to hydrolyse urea. Two carbon isotopes are currently used for the UBT, either $^{13}$C, which is a non-radioactive isotope, or radioactive $^{14}$C. Carbon-labelled urea is given to the patient. In the presence of *H. pylori* urease, the urea is hydrolysed and labelled carbon dioxide is absorbed into the bloodstream and exhaled in the breath. Breath samples are collected by blowing into a tube and the amount of $^{13}$CO$_2$ or $^{14}$CO$_2$ is measured by a mass spectrometer or a scintillation counter, respectively. A distinct advantage of this test is that a positive UBT result indicates current infection.

A European standard protocol has been developed for the $^{13}$C-UBT (Logan et al., 1991). Although some results indicate that the test is accurate under acid
suppression, when the test includes a meal including citric acid (Leodolter et al., 1999), the sensitivity and specificity of the UBT is generally very high and in clinical practice this test is particularly useful for the assessment of *H. pylori* eradication (Goddard and Logan, 1997; Gisbert and Pajares, 2004). However, false negative results may occur if the patient has recently received treatment with antibiotics, bismuth or proton pump inhibitors (PPI). PPI's suppress *H. pylori* infection and may give rise to false negative UBT results (range 17-61%) if the test is performed within one week of treatment (Logan et al., 1995; Savarino et al., 1999).

### 1.1.4.2.2 Antibody Detection

*H. pylori* infection induces both local and systemic immune responses in the host, the latter yielding serum antibodies mainly of the IgG1 subclass that are directed against the outer bacterial surface proteins, the flagella and other bacterial proteins such as urease. Due to the chronic nature of the infection and the constant systemic IgG response induced, numerous serologic tests are currently available to detect the antibodies produced. However, their application in a given population may require local adjustment of cut-off values (Harris et al., 2005). Also the differences in accuracy observed with these tests may be due to the different antigen preparations used. Poor specificity has been observed with the first commercially available kits due to cross reactivity of *Helicobacter* flagellar antigens with those of *Campylobacter* species (De Boer, 1997). New second-generation kits employ a mixture of purified antigens and these increase specificity (Breslin and O'Morain, 1997).

Serology is not recommended for post-treatment assessment of *H. pylori* status as IgG levels may take six months or longer to fall to levels within the uninfected
range following treatment success. Serology may be useful for monitoring the long-term effect of treatment in the clinical trial setting, although 2% of *H. pylori*-infected individuals do not mount a systemic IgG response (Méraud, 1996). It is important to test simultaneously the pre- and post-treatment sera and to demonstrate a fall in antibody titre rather than seronegativity. A titre reduction of > 50% is usually a reliable indicator of eradication as titres differing by ±20%, irrespective of treatment success, may merely represent *H. pylori* suppression (WPEHPSG, 1997).

Several studies investigated the usefulness of serologic markers as predictors of the gastric mucosa status. Serum anti-parietal cell antibodies were found to correlate with antral atrophy (Lo et al., 2005), and serum pepsinogen I/II ratio was inversely related to the grade of corpus atrophy (Graham et al., 2006). An other study showed that the combination of anti-*H. pylori* antibodies, serum pepsinogens I and II, and gastrin-17 could be used not only to identify patients with atrophic gastritis but also to localize the site of atrophy (Germana et al., 2005). In other studies, anti-*H. pylori* antibodies combined with serum pepsinogens I and II (Watabe et al., 2005) or the level of *H. pylori* IgG2 antibodies (Ren et al., 2005) appeared to be a predictive marker for the development of gastric carcinoma.

1.1.5 Geographic variation

Phylogenetic analyses based on sequence comparisons have shown that distinct alleles of *H. pylori* genes exist in different geographical regions. Since *H. pylori* is transmitted mainly within families, it is therefore likely that the geographic variations stem from *H. pylori* strains that have evolved over long
periods of time in different geographical locations. The distinct allele types of *H. pylori* genes found in isolates from various regions are thought to reflect either the original founder population that was first introduced in the particular area, or the selection for antigenic or functional variants with growth and persistence advantages in a particular host population (Suerbaum and Achtman, 2004). By studying *H. pylori* sequence data from both housekeeping genes and virulence associated genes from isolates belonging to different geographical locations, East Asian strains were found to cluster, as did the alleles of African strains, whereas strains from Europe, America and Australia formed a heterogeneous third group (Achtman et al., 1999). Similarly Kersulyte and colleagues analyzed more than 500 strains of *H. pylori* from five continents and found that when studying genetic subtypes based on variations in the cag PAI, cagA and vacA clustered according to geographical origin (Kersulyte et al., 2000).

1.1.6. Genomic variability

Much attention and research effort has been spent on the remarkable genetic diversity between isolates from different individuals. It is surprisingly difficult to find two isolates with identical genomic fingerprints by methods such as random amplification of polymorphic DNA (RAPD) and restriction fragment length polymorphism (RFLP) analysis (Akopyanz et al., 1992a; Akopyanz et al., 1992b; Fujimoto et al., 1994; van Doorn et al., 1998). In addition, comparison of the genomes of two strains has shown that 7% of the genes are specific for each strain (Alm et al., 1999). The mechanisms which confer this diversity include point mutations, chromosomal rearrangements, strain specific restriction-modification systems, horizontal gene transfer between strains, impaired DNA repair mechanisms, and an exceedingly high frequency of recombination (Blaser and
Berg, 2001). The recombination rate in *H. pylori* exceeds that of all other studied bacteria and it can be demonstrated by comparing different clones from a single patient (Falush et al., 2001; Israel et al., 2001; Kuipers et al., 2000). The *H. pylori* is naturally competent for transformation, which allows uptake of DNA from neighboring strains in the local environment followed by recombination events for integration of alien DNA into the chromosome. *H. pylori* have also been shown to possess a conjugation-like mechanism for DNA uptake (Nedenskov - Sorensen et al., 1990). Hofreuter and colleagues showed that this natural transformation competence is mediated by proteins encoded by the ComB gene cluster (Hofreuter et al., 2001). Uptake of foreign DNA and subsequent homologous recombination would result in genetic diversity, and generate new potentially beneficial genotypes more rapidly compared to spontaneous mutations. Interestingly, the average size of recombined fragments in *H. pylori* (417 bp) is much smaller compared with other bacteria (Falush et al., 2001).

Mechanisms for generation of diversity may be responsible, at least in part, for the ability of *H. pylori* to survive in the harsh gastric environment where it encounters continuous selection pressures, such as local changes in mucosal expression of Lewis antigens, host immune and inflammatory responses. In addition differences between individual host traits that effect *H. pylori* growth and persistence will lead to further selection pressure during each new infection (Blaser and Berg 2001). Thus, development of variation within the *H. pylori* population followed by continuous selection for sub-clones with improved fitness for individual hosts over years or even decades of infection, has led to an exceedingly diverse and still most host-adapted bacterial species.
1.1.7. Treatment of *H. pylori* infection

Treatment of *H. pylori* infection is strongly recommended in patients suffering from peptic ulcer disease and MALT-lymphoma. John Lykoudis was a general practitioner in Greece who treated patients from peptic ulcer disease with antibiotics long before it was commonly recognized that bacteria were a dominant cause for the disease (Rigas *et al*., 1999).

It is advisable to treat *H. pylori* infection in patients with functional dyspepsia and in those with a family history of gastric cancer, but it is not advised as prevention of gastric cancer in otherwise healthy, asymptomatic subjects (EHPSG, 1997; Tytgat, 1998). It was reported that recurrence of peptic ulcer disease was completely prevented after successful treatment of the infection, and as the reinfection rates are low, unsuccessful eradication is usually responsible for recurring infections (van der Hulst *et al*., 1997). Triple therapy during one week with a combination of two antibiotics (clarithromycin or amoxicillin and metronidazole) and a proton pump inhibitor, PPI (omeprazole, lansoprazole) give the highest eradication rates (Hunt and Lam, 1998). The shorter the treatment time, the higher is the compliance, which is of special importance for the treatment of *H. pylori* infection in children (Oderda, 1998). If the triple therapy fails, quadruple therapy based on PPI plus classic bismuth based triple therapy (bismuth, metronidazole and tetracycline) can be used (Graham *et al* 1996). Treatment failures of *H. pylori* may be due to poor patient compliance, antimicrobial resistance or failure of drug delivery across the gastric mucosa (Goddard, 1998). The addition of proton pump inhibitors to the therapy of *H. pylori* infection improves ulcer healing, increases eradication rates and decreases primary resistance, since the antibiotics are more effective when the intragastric pH is
raised (Scott et al., 1998; Lind et al., 1999). Metronidazole crosses the gastric mucosa by simple diffusion and is very stable in gastric juice. Amoxicillin crosses the mucosa very poorly and is fairly stable at low pH but needs a higher pH to be effective in vivo. Clarithromycin is actively transported from blood to gastric lumen and is quite unstable at lower pH. The use of PPI together with these antimicrobial agents improves *H. pylori* eradication.

Several studies were performed to study the effect of PPI on *H. pylori* strain selection and distribution of bacteria in the stomach (Hazell 1997). If more pathogenic strains of *H. pylori* are selected during therapy, the risk of gastric cancer may be enhanced in subjects taking these therapies for long periods of time. New combinations of "old" antimicrobial agents or new antimicrobial agents may be an option for eradication of *H. pylori* (Tytgat, 1999). Long acting antibiotics and acid suppressants may reduce the length of therapy and increase patient compliance. Single capsules containing combinations of triple therapy constituents, the linking of two drugs into one, or the use of "designer drugs" based on new knowledge about the *H. pylori* genome and the organism's life may also improve treatment. Designer drugs that inhibit the action of specific proteins for survival of *H. pylori* in the gastric mucosa or more effective acid suppressants may also become available.

### 1.1.8. Antimicrobial resistance

Bacterial resistance to different antibiotic compounds is measured by minimal inhibitory concentration (MIC) values, which give a measurement of the bacterial susceptibility or resistance to certain antibiotics. The laboratory techniques for determination of MIC values are disk diffusion, agar dilution, and
the E-test (Glupczynski et al., 1991; Valdez et al., 1998). The elliptic inhibitory zone on bacterial growth caused by the E-test strip on an agar plate indicates the in vivo susceptibility of the microorganism. Differences in antibiotic resistance have been observed between clones of genotypically identical isolates of *H. pylori* (Dore et al., 1998). Antibiotic resistant organisms, a resistant infection even though the organisms are sensitive in vitro, or both, may contribute to failures in *H. pylori* eradication therapies (Graham, 1998). Intracellular activity of the antibiotic is of importance for a successful eradication of *H. pylori* in vitro, indicating that intracellular organisms may be responsible for recurrent infections after treatment (Hultén et al., 1996).

Antimicrobial resistance in *H. pylori* is emerging, especially resistance to nitroimidazoles (metronidazole), and is now as high as 10-50% in certain countries in Europe and the USA (Mégraud, 1998; Mégraud and Doermann, 1998; Wolle et al., 2002; Elviss et al., 2005 Chisholm et al., 2007; Boyanova et al., 2008). In developing countries, almost all of the isolated strains are resistant to metronidazole. The high resistance to metronidazole in developing countries has been linked to the use of this drug in treatment of parasitic infections and with their use for treatment of dental and vaginal infections in developed countries. The decreased ability of the bacteria to reduce the nitro group on the compound, a reduction which is needed for the drug to be effective in breaking the DNA strand, is thought to be responsible for resistance to these compounds. In patients with recurrent infection, it was found that metronidazole resistant and sensitive *H. pylori* organisms coexisted in biopsies before and after treatment (Weel et al, 1996; Jorgensen et al., 1996). Up to 15% of isolated strains from developed countries are resistant to the macrolide clarithromycin. The secondary resistance,
which occurs after eradication therapy, may increase to up to 60%. Resistance to clarithromycin is caused by a mutation in one of the two copies of the 23S rRNA gene which can be detected by PCR followed by RFLP of the amplified product, and the resistance is stable by subcultivation of *H. pylori* in vitro (Méraud, 1998; Hultén et al., 1997).

A assay to detect clarithromycin resistance directly from biopsies showed good results for detection of multiple genotypes, sensitive and resistant *H. pylori*, when present in the same sample (Marais et al., 1999). Resistance to amoxicillin is very rare, but differences in susceptibility are related to absence of certain penicillin binding proteins in the bacteria. After triple therapy with omeprazole, amoxicillin and metronidazole, a genetically identical strain as the primary isolate was responsible for recurrence of infection (Adamsson et al., 1999).

### 1.1.9 Development of Vaccines

To date, *H. pylori* is one of the most common infections faced by mankind. Infection usually occurs during childhood, and when left untreated results in lifelong colonization of the stomach. Antimicrobial therapy is currently the method of choice for the treatment, however, complex dosing, inconsistent efficiency, development of antibiotic resistance, costs and side effects compromise their widespread use. As a result, it is important to explore new strategies for the prevention and eradication of *H. pylori*. Development of an effective vaccine would be useful since it will be both effective and economic in use. In trials so far, potential vaccine candidates such as whole-cell extracts or sonicates of *H. pylori* containing a mixture of immunogenic outer membrane proteins, porins and adhesions, or *H. pylori* urease, catalase, VacA and heat shock proteins together
with cholera toxin (CT) or *E. coli* labile toxin (LT) as adjuvants, have been tested in animal models with variable, but optimistic results (Saldinger et al., 1997). No adjuvant has yet been found suitable for use in humans, and without the adjuvant, the vaccine candidate works poorly giving a low and insufficient immune response. For a vaccine to be effective, the antigen needs to be universal among strains of *H. pylori*, and the delivery of the antigen must give a suitable response in the host without any side effects induced by the adjuvant. Sonicates of *H. pylori* together with Freund’s incomplete adjuvant (parenteral) or *E. coli* LT (oral) were tested in a gnotobiotic piglet animal model (Eaton et al., 1989).

After challenge with *H. pylori*, the vaccination suppressed, but did not prevent, the infection and the vaccination did not cure the infection in piglets already colonized by *H. pylori*. Oral administration of recombinant urease and LT to Rhesus monkeys protected against *H. pylori* infection and did not give any side effects (Dubois et al., 1999). A vaccine study with orally administered urease and LT to *H. pylori* infected human volunteers have been performed with similar results, the vaccine is immunogenic in humans and the combination of urease and LT is well tolerated (Michetti et al., 1999). The antigen, which may be included in a final vaccine used in humans, needs to be effective when used as a single component, it should give an additive or synergistic effect together with urease, it needs to be easily produced in large scale and easily purified in large amounts. It needs to be non toxic and should not give cross-reactions with human tissue antigens, and it needs to be conserved among *H. pylori* isolates from different geographical regions. *H. pylori* LPS which contains cross-reactive Lewis antigens, and the CagA protein which is only produced by a subset of strains, are therefore not useful in a vaccine. Future vaccine candidates may be found by proteome techniques, including two-
dimensional gel electrophoresis (McAtee et al. 1998; Sutton and Doidge, 2003; Aebischer et al., 2008). The antigen may be introduced into humans by a live carrier organism (live carrier vaccination) or by intramuscular injections of *H. pylori* DNA for specific genes such as urea, which diminishes the need of mucosal adjuvants to obtain immune responses in the host. The routes of vaccine administration are also of importance. Oral, nasal and rectal administration has been tested with positive results. For a vaccine to be successful, not only prophylactic but also therapeutic applications need to give positive test results.

1.2. Consequences of *H. pylori* Infection

1.2.1. *H. PYLORI* INFECTION AND GASTRIC DISEASES

The discovery of *H. pylori* in 1982 (Warren & Marshall 1983) was the starting point of a revolution concerning the concepts and management of gastro duodenal diseases. The prevalence of *H. pylori* infection and disease progression varies both within and between countries (Al-Moagel et al., 1990; Graham et al. 1991; Kneller et al., 1992; Mitchell 2001; Hussein et al., 2008). Developing countries have a higher prevalence of infection than developed countries. Interestingly, infected individuals from developing countries have a higher probability of developing gastric ulcer (GU) disease, while in developed countries they are more likely to develop duodenal ulcer (DU). It is thought that these differences occur due to a number of factors such as socioeconomic status, diet, and genetic predisposition (Mitchell 2001). Infection with *H. pylori* usually occurs during childhood and lasts for life unless treated. In spite of the fact that there is clear histological evidence for lifelong gastritis in almost all infected individuals, a large proportion of those colonized with *H. pylori* remain asymptomatic. Probably less than 15% of all infected people develop serious gastro duodenal pathologies such as gastric or
duodenal ulcers, gastric adenocarcinoma and lymphoma (Howden 1996). *H. pylori* infection has been implicated in the pathogenesis of many extragastric conditions ranging from atherosclerosis to skin diseases, but documentation is poor and the associations are controversial (Leontiadis *et al.*, 1999; Franceschi and Gasbarrini, 2007). Although colonization of the human gastric mucosa by *H. pylori* inevitably results in persistent inflammation, development of peptic ulcer disease or gastric malignancy is observed only in a minority of those colonized. Despite this less than perfect association between *H. pylori* and the development of gastric malignancies, *H. pylori* classified as a type I carcinogen (IARC 1994).

The observed variation in disease outcome is currently thought to be mediated by an intricate interplay between bacterial, host and environmental factors (Sgouros and Bergele, 2006). On the bacterial side there is substantial phenotypic and genotypic diversity between *H. pylori* isolates, both at the genomic level and at the level of individual virulence factors. Strain to strain genetic variability in bacterial virulence factors affects the ability of the organism to colonize its host and to induce subsequent disease. Conversely, on the host side, *H. pylori*-associated gastric disease in part results from an inappropriate host immune response to the chronic presence of *H. pylori* infection. This is reflected by the host gene polymorphisms in key cytokine genes, such as in the IL-1β and IL-1β receptor antagonist encoding genes that were shown to correlate with an increased risk for the development of gastric carcinoma (El-Omar *et al.*, 2000). Finally, the environmental factors resulting from differences in lifestyle, eating and drinking habits, smoking, and use of drugs such as non-steroidal anti-inflammatory drugs (NSAIDS) and proton pump inhibitors (PPIs) all affect the gastric
microenvironment, and thus disease risks (Correa et al., 1998; Koivisto et al., 2008; Papa theodoridis et al., 2006).

1.2.1.1 Gastritis

The healthy, uninfected human stomach contains very few immune and inflammatory cells, initial colonization by H. pylori results in an acute inflammatory response (acute gastritis), which is characterized by infiltration of lymphocyte into the gastric mucosa. The acute infection is also accompanied by transient hypochlorhydria, i.e. reduced gastric acidity. If these initial responses fail to clear the infection, there is a gradual accumulation of neutrophils, T cells, B cells and macrophages into the gastric mucosa. After a few weeks, there is a massive invasion of the tissue by immune and inflammatory cells, which is a characteristic histological picture of chronic active gastritis. The continuous presence of H. pylori elicits a local mucosal IgA antibody response and a systemic humoral response, neither of which can eradicate the infection. Once established, the infection persists for the lifetime of the host if not eradicated with antibiotics. In some cases, it progresses to severe gastric diseases such as duodenal ulcer, gastric ulcer, gastric atrophy, and gastric carcinoma. However, in the great majority of infected individual the H. pylori-related chronic gastritis is asymptomatic (Dixon, 2001). The self-inoculation experiment by Marshall and Morris provided evidence that H. pylori infection causes gastritis (Marshall et al., 1985; Morris and Nicholson, 1987).

1.2.1.2 Peptic ulcer

H. pylori infection is closely linked to peptic ulcer. Approximately 90%-95% of duodenal ulcers and 70%-75% of gastric ulcers are attributable to infection with H. pylori (Covacci et al., 1999). Use of non-steroidal anti-inflammatory drugs
(NSAID) is an increasing cause of peptic ulcer and, as the frequency of *H. pylori* infection declines, it will become the most common cause. It is now estimated that the lifetime risk of developing peptic ulcer disease in *H. pylori* infected individuals is approximately 10% (Parsonnet, 1995). The strong link between *H. pylori* and peptic ulcer, and the observation that cure of the infection results in cure of the ulcer disease, provided strong evidence that *H. pylori* is etiologic in the pathogenesis of peptic ulcer disease. Compelling evidence that *H. pylori* is a cause of recurrent ulcers comes from the observation that the recurrence rate of duodenal ulcers is markedly reduced after successful treatment of *H. pylori* infection (Rauws and Tytgat, 1990; Miehlke et al., 1995). Moreover, iatrogenic infection of patients in which a natural *H. pylori* infection has been previously cured by antimicrobial treatment can lead to ulcer recurrence (Langenberg et al., 1990). Thus in the presence of this overwhelming evidence, *H. pylori* infection is accepted as a cause of ulceration. A unifying hypothesis relates increased duodenal acid load, *H. pylori*, and duodenal ulcer disease (Graham, 1989). The critical factor is that lowering the pH in the duodenum below the pKa for bile acids would remove the bile acids that normally inhibit growth of *H. pylori*. The abnormalities in gastro duodenal physiology associated with *H. pylori* infection all increase duodenal acid load. Other factors that may also increase the duodenal acid load are smoking and stress. The corollary is that reducing the duodenal acid load with antisecretory drugs or antacids not only would help accelerate ulcer healing, but also would make the environment in the duodenal bulb inhospitable for the growth of *H. pylori*. An apparent paradox is that two *H. pylori*-associated diseases appear to be mutually exclusive. The presence or history of duodenal ulcer disease protects against development of gastric cancer. The very low risk of gastric cancer in patients with duodenal ulcer has been confirmed in
epidemiologic studies evaluating the risk of *H. pylori* infection for the development of gastric cancer (Hansson et al., 1996).

### 1.2.1.3. Gastric cancer

Gastric cancer remains a major cause of mortality and morbidity worldwide, and the total number of gastric cancer cases is predicted to rise as a result of population growth (Lochhead and El-Omar, 2008). Globally, gastric cancer is the second most common cause of cancer-related death, the predicted incidence for 2010 is 1.1 million, and the majority of this health burden would be borne by economically lesser-developed countries (Parkin, et al., 2006). The association between gastritis and gastric cancer was recognized decades before the discovery of *H. pylori* as a cause of gastritis. The link of *H. pylori* infection was confirmed after the discovery of the pathogenic role of the organism in gastritis (Graham, 2000). Although the incidence of gastric adenocarcinoma has steadily declined for the past 50 years, gastric cancer was the most common cancer in the early 20th century and remains the second most frequent cancer related death in the world (Peura, 1996; Suerbaum and Michetti, 2002). In 1994, the World Health Organization’s (WHO) International Agency for Research on Cancer classified *H. pylori* as a definite carcinogen. This classification was largely based on the epidemiologic links and not on a specific pathway. Gastric cancer is typically separated histologically into two types: an intestinal form strongly related to *H. pylori* and the diffuse form where the association is positive but weaker (Peek and Blaser, 2002). The incidence of gastric cancer is consistent with a high incidence of *H. pylori* infection as the cause of the precursor lesions, chronic atrophic gastritis (Correa, 1996). Several case-controlled studies have shown that *H. pylori* seropositivity is associated with increased risk of gastric cancer (2.1 — 16.7- fold
greater than in seronegative persons (Barreto et al., 1997; Peek and Blaser, 2002; Misra et al., 2007).

Prospective studies have demonstrated that the longer the time interval between *H. pylori* detection and gastric cancer diagnosis, the higher the risk of developing cancer. In the EUROGAST Study Group, *H. pylori* increased the risk of gastric cancer by a factor of 6. A prospective study from Japan illustrates that in follow-up of approximately one decade, no gastric cancer developed in patients without *H. pylori* or patients whose infection was eradicated (Uemura et al., 2001). Cancer development in the course of *H. pylori* infection is typically multifactorial, acute gastritis is the initial lesion, progressing in some patients to multifocal atrophic gastritis. The sequence of events leading to the intestinal type of gastric cancer as a consequence of *H. pylori* infection includes the development of superficial gastritis which then can progress to atrophy, intestinal metaplasia, dysplasia and finally invasive carcinoma (Correa et al., 1975). The extent of intestinal metaplasia seems to be a crucial predictor of cancer, but it may simply be a marker for the presence of low acid secretion or the extent of atrophy (Lechago and Correa, 1993). The risk to the population can be increased by the behaviour that promotes cancer (e.g., cigarette smoking or a high a salt diet) or decreased by consumption of fresh fruit and vegetable (Hwang et al., 1994). The highest rates of gastric carcinoma are found in populations that have an accelerated acquisition of chronic atrophic gastritis, so that patients with severe multifocal atrophic gastritis have over a 90 fold greater risk of developing adenocarcinoma than those with normal mucosa (Sipponen and Marshall, 2000).
Although chronic inflammation is a sufficient cause for progressive damage and loss of cellular element other explanations are also possible including one based on molecular mimicry (Appelmelk et al., 1997; Negrini et al., 1996). The molecular mimicry involved mucin glycoproteins and a study had shown that H. pylori expresses α side chain polysaccharides with blood group determinants including Le^x and Le^y (Appelmelk et al., 1996). Molecular mimicry leads to the synthesis of autoantibody and following that development of atrophy (Appelmelk et al., 1997; Appelmelk et al., 1996).

1.2.1.3. Gastric Lymphomas

H. pylori play a crucial role in the pathogenesis of primary gastric B cell lymphoma (MALT Lymphoma) (Zucca et al., 1998). The association between gastric MALT lymphoma and chronic gastritis proceed the discovery of H. pylori and the association has been confirmed by demonstration of strong epidemiologic link between H. pylori and gastric MALT lymphoma (Parsonnet et al., 1994). The normal stomach lacks organized lymphoid tissue, persistent antigen stimulation from the by products of chronic H. pylori infection induces lymphoid tissue (Sorrentino et al., 1996). In 70 - 80% of cases regression of MALT lymphoma is observed after eradication of H. pylori (Wotherspoon et al., 1993)

1.3. Pathogenic mechanisms of H. pylori infection

1.3.1 H. pylori virulence factors

H. pylori-induced gastro duodenal pathogenesis depends on the persistence of the infection, the production of specific virulence factors that cause damage to gastric epithelial cells and disruption of the gastric mucosal barrier, and the inflammatory response of the host (Kusters et al., 2006). So far, there is no evidence that causing disease would facilitate transmission of H. pylori infection
from one individual to another, or provide other benefits to the bacteria. Nevertheless, some strains are considered distinctly more pathogenic than others. Unfortunately, the huge number of people infected by *H. pylori* prohibits treatment of every person since the great majority will never develop *H. pylori*-related symptoms of disease. Consequently, much research has been focused on definition of markers for strains associated with development of peptic ulcer disease and gastric cancer (Ando *et al.*, 2002; Lehours *et al.*, 2004; Oleastro *et al.*, 2006; Lu *et al.*, 2005; Rhead *et al.*, 2007; Yamaoka *et al.*, 2002). To date the best-recognized markers of *H. pylori* pathogenicity are the *cagA*, *vacA*, *iceA* and *babA* genes.

### 1.3.1.1. Urease

*H. pylori* contain urease, an enzyme abundantly produced by these bacteria. It is a significant survival factor for *H. pylori*, since ammonium (NH4+) is produced from urea in the stomach. NH4+ neutralizes HCL and allows *H. pylori* to colonize the gastrointestinal tract. NH4+ concentration in the stomach of infected patients is significantly higher than in non-infected subjects (Thomsen *et al.*, 1989). Similarly, NH4+ levels are higher in infected patients than in the same patients following *H. pylori* eradication (Chittajallu *et al.*, 1991). There are a number of toxic effects directly resulting from NH4+ within the bowel, including, DNA synthesis disorders, increased risk for viral infection, and carcinogenesis (Méraud *et al.*, 1992). Furthermore, a decrease in oxygen use by gastric cells has been reported in cultures (Tsujii *et al.*, 1992). In vitro, the high levels of NH4+ generated by *H. pylori* have a significant effect on reduced cell viability, an effect unseen in the absence of urea and in strains with non-functional urease. These effects are seen to be reversible when urease is added in the medium, and the number of
viable cells diminishes (Mégraud et al., 1992). Urease is essential for colonization, as has been demonstrated in experiments using *H. pylori* strains with non-functional urease (Eaton et al., 1991). These strains are unable to colonize under hypochlorhydria conditions (Eaton et al., 1994), which demonstrates that urease is essential for survival regardless of its role in gastric acid neutralization. On the other hand; major histocompatibility complex II (MHC II) molecules regulate immune response through antigen presentation to CD4 T cells.

The binding of *H. pylori* to MHC II through urease has been examined, and results increasing gastric cell apoptosis (Fan et al., 2000). Apoptosis induction is dependent upon MHC II expression, and may become eventually blocked when anti-MHC II antibodies are used; *H. pylori* do not indicate apoptosis in cells deficient in MHC II expression (Fan et al., 2000).

### 1.3.1.2. The cag Pathogenicity Island

The CagA (cytotoxin-associated gene A) was so named because it was thought to be associated with expression of the Vacuolating cytotoxin VacA. However, the *cagA* gene is not chromosomally linked to the *vacA* gene; nor is it needed for expression of VacA (Xiang et al., 1995). The *cagA* gene is present in strains with enhanced virulence, and has been identified as an important risk factor for development of severe gastric disease. *H. pylori* strains are divided into two groups including type I and type II strains, based on whether or not they express *cagA* gene (Xiang et al., 1995). The *cagA* gene is located in a ~40 -Kbp *cag* Pathogenicity island (*cag PAI*) which contains about 30 genes (Censini et al., 1996). The GC content of the *cag PAI* differs from the rest of the *H. pylori* chromosome, which suggests that it originated from an ancient event of horizontal
gene transfer (Covacci and Rappuoli, 2000). The cag PAI encodes the components of a needle-like structure, which is involved in transport of CagA from the bacterial cell directly into the cytoplasm of the host cell (Stein et al., 2001). Inside the host cell, CagA undergoes tyrosine phosphorylation by the host cell kinases and then induces dephosphorylation of the host cell proteins (Asahi et al., 2000; Odenbreit et al., 2000; Stein et al., 2000). Once phosphorylated, CagA interacts with different components in the host cell signal transduction pathways and the actin binding protein cortactin, which ultimately affect cytoskeletal organization (Selbach et al., 2002). A study showed that cagA positive bacteria, but not cagA negative bacteria, induce a “hummingbird”-like phenotype to AGS and MDCK cell lines. This phenotype is characterized by out spread and elongated growth of the cell and the presence of finger-like protrusions containing bundles of actin filaments (Segal et al., 1999). It has also recently been shown that CagA could associated with epithelial tight-junction proteins, which lead to disruption of epithelial barrier function and dysplastic alterations in epithelial cell morphology (Amieva et al., 2003; Bagnoli et al., 2005; Bourzac et al., 2007).

1.3.1.3. The Vacuolating cytotoxin (VacA)

All H. pylori strains have a vacA gene coding for cytotoxin that induces epithelial cell vacuolization (Cover et al., 1994; Harris et al., 1996), but only 50 to 65% of H. pylori strains produce the cytotoxic protein. Infection by toxin-producing H. pylori strains has been seen to be more frequent in patients with peptic ulcer (Atherton et al., 1997; Hou et al., 2000) and gastric cancer (Ricci et al., 1996; Rudi et al., 1998) when compared to patients with only gastritis. Two polymorphic regions have been documented within vacA (Atherton et al., 1995). One is on the second half of the signal sequence (s1a/s1b/s1c or s2), the other on the central
region \((m1 \text{ or } m2)\). The type \(s1\) vacA produces a fully active cytotoxin, whereas type \(s2\) vacA produces a toxin with a hydrophilic N-terminal extension that blocks vacuolating and pore-forming activities (Atherton et al., 1995).

Sequence \(s1\), but not sequence \(s2\), is closely linked to the cytotoxin's in vitro activity, peptic ulcer, and the presence of gene \(cagA\) (Atherton et al., 1995; Atherton et al., 1997). In contrast, \(cagA\) is not associated to cytotoxicity in epithelial cells, as has been seen in a study following \(cagA\) gene mutation (Tummuru et al., 1994). Therefore, the presence of \(cagA\) is not essential for cytotoxicity induction. In contrast to \(s1\) strains, \(s2\) strains only express the VacA protein in small amounts, and only exhibit mild cell vacuolization (Rudi et al., 1998). In this regard, it has been postulated that the clinical progression of \(H. pylori\) infection is dependent upon the vacA genotype. \(H. pylori\) strains possessing the \(s1/m1\) type of vacA demonstrate enhanced cytotoxic activity compared to \(s1/m2\) strains, and are epidemiologically associated with gastric carcinoma (Atherton et al. 2001; Zambon et al., 2003).

Recently a third polymorphic determinant of vacuolating activity has been discovered which is located between the signal region and midregion, which is termed the intermediate (i) region. Two i-region types were identified, \(i1\) and \(i2\); and \(i1\)-type strains were reported to be strongly associated with gastric adenocarcinoma in Iranian patients and it was hypothesized that vacA i-region typing may provide an improved predictor of \(H. pylori\) strain carcinogenic potential (Rhead et al., 2007). However other studies conclude that vacA genotypes with a combination of 3 regions also failed as a disease determinant in the East Asian
and Southeast Asian countries where gastric cancer is a major clinical problem (Ogiwara et al., 2007).

A study has shown that VacA accumulates in clusters on the bacterial surface. During *H. pylori* adherence to host cells, the toxin clusters are directly transferred in active form from the bacterial surface to the host cell surface, followed by uptake (Ilver et al., 2004). The mechanism through which VacA induces vacuolization is only partially understood. This protein is thought to be hexameric, and its assembly is favored by an acidic pH, resulting in an anion-selective channel across the cell’s lipid bilayer (Cover et al., 1997; Iwamoto et al., 1999). It is then translocated to the cytosol, where it interferes with the vesicular transit of lysosomes, and may again set up an ion channel across endosomal membranes, which is partly responsible for the vacuolization process (Montecucco et al., 1999).

1.3.1.4. The *iceA*

An independent strain-specific *H. pylori* locus associated with disease is *iceA* gene (Peek et al 1998), which exists in two major allelic sequence variants, *iceA1* and *iceA2*. Induction of *iceA1* is dependent on bacterial contact with epithelial cells. The deduced *H. pylori* *iceA1* product demonstrates strong homology to a restriction endonuclease; however, mutations found in the majority of *iceA1* sequences preclude translation of a full-length homolog. In contrast to *iceA1*, *iceA2* has no homology to known proteins, and its structure reveals patterns of repeated peptide cassettes. *H. pylori* *iceA1* strains are significantly associated with the presence of peptic ulceration and distal gastric adenocarcinoma (Kidd et al 2001; Peek et al 1998), and levels of *iceA1* expression within colonized human
gastric mucosa are directly related to the severity of acute inflammation and IL-8 expression (Peek et al 2000; Takeuchi et al 2002).

The iceA1 gene is associated with the presence of cagA and the vacA s1 allele, but is only found in 25% of H. pylori isolates from the United States, which approximates the percentage of infected persons who progress to peptic ulceration and/or gastric cancer (Peek et al 1998). The association of iceA1 with increased tissue damage and disease, its allelic distribution in a minority of clinical strains, its linkage disequilibrium with cagA and vacA s1 alleles, and its induction by physiologic events that induce pathologic responses (adherence) collectively suggest that iceA1 may be a marker for strains that induce more severe gastric inflammation and injury. In a recent study, the prevalent circulating genotypes in chronic gastritis and duodenal ulcer were cagA, vacA s1m2, iceA2, and cagA, vacA s1m2, iceA1 genotypes, respectively. In addition, it was found that cagA, vacA s1m2 genotype seems to be a common virulence factors in both chronic gastritis and duodenal ulcer while iceA alleles show specificity for gastro duodenal pathologies (Caner et al., 2007). Similarly other study shows that patients infected by H. pylori cagA(+), vacAs1 m1 and iceA1 genotype showed higher levels of oxidative DNA damage than patients infected with H. pylori cagA(-), vacAs2 m2 and iceA2 genotypes and uninfected patients (Ladeira et al.,2008). However in another study neither single genes nor combinations of vacA, cagA, cagE, iceA, and babA2 genes was significantly helpful in predicting the clinical outcome of H. pylori infection and the high prevalence of these genes in H. pylori isolated from such patient groups suggests that H. pylori strains are geographically dependent (Chomvarin et al., 2008).
1.3.1.5. The Outer Membrane Protein (OMPs)

In Gram negative bacteria, the outer membrane mediates the interaction with their surroundings. During infection, proteins present on the outer membrane of *H. pylori* are assumed to be altered in such a way that recognition by the host immune system is minimal (van Amsterdam *et al.*, 2006).

The sequence analysis of the genomes from the two completely sequenced *H. pylori* strains 26695 and J99 has revealed that an unusually high proportion of identified open reading frames are predicted to encode outer membrane proteins (OMPs), and consequently, recent attention has been directed toward a possible role of these OMPs in *H. pylori* pathogenesis (Solnick *et al.*, 2004). BabA is a membrane-bound adhesin encoded by the *H. pylori* strain-selective gene *babA2* that binds the blood-group antigen Lewis\(^b\) present on gastric epithelial cell membranes, and *H. pylori* strains possessing *babA2* are associated with increased risk for gastric adenocarcinoma (Gerhard *et al.*, 1999).

Another adherence-related OMP is HopQ, encoded by a gene that has been shown to co-vary with the cag pathogenicity island. Two distinct families of *hopQ* alleles have been identified. Type I alleles are found significantly more commonly in *H. pylori* cag+vacA s1 strains isolated from ulcer patients than in cag– vacA s2 strains harvested from patients without ulceration (Cao and Cover 2002), suggesting that this locus may have an important role in virulence.

The sialyl-dimeric-Lewis\(^x\) glycosphingolipid has been demonstrated to function as a receptor for *H. pylori* in vivo (Mahdavi *et al.*, 2002), the *H. pylori* adhesin required for binding was identified as SabA, and since sialyl-Lewis\(^x\) antigens are established tumor antigens and markers of gastric dysplasia that are upregulated by chronic
gastric inflammation, these results further emphasize the pivotal role of *H. pylori* adherence in the induction of gastric inflammation and injury.

Another *H. pylori* outer membrane protein that may mediate disease is a proinflammatory protein encoded by *oipA* (Yamaoka *et al* 2000). The *oipA* gene is present in virtually all *H. pylori* isolates, but its expression is modulated by phase variation via slipped strand mispairing of a variable number of CT-dinucleotide repeats in the 5'-region of *oipA* (Yamaoka *et al*., 2000). The vast majority of *cag*+ strains isolated from patients in East Asia have an in-frame copy of *oipA*, and when co cultured with gastric epithelial cells in vitro, these strains were found to induce high levels of IL-8. Inactivation of *oipA* decreased IL-8 levels by ≥40% (Yamaoka *et al*., 2000), whereas inactivation of both *oipA* and *cagE* in the same strain completely abolished IL-8 production. *H. pylori* strains that contain a functional copy of *oipA* are linked with more severe gastric inflammation, higher bacterial colonization density, enhanced mucosal levels of IL-8, and duodenal ulcer disease (Yamaoka *et al* 2002). In a murine model of *H. pylori*-induced gastritis, the ability of *H. pylori* to successfully colonize C57Bl/6 mice was shown to be dependent on the in-frame status of OipA, and infection with an isogenic *oipA* mutant led to a significant decrease in the severity of gastritis as well as a reduction in chemokine production compared to levels induced by the parental wild-type *H. pylori* strain (Yamaoka *et al* 2002a), indicating a potential role of OipA in *H. pylori* pathogenesis.

The adhesins encoded by *oipA*, *hopZ*, *hopO*, *hopP* and *BabB* are all thought to be regulated by phase variation through slipped strand repair mechanisms. Individually, these adhesins are not essential for colonization. However, the
expression of two or more of these proteins enhances the ability of *H. pylori* to colonize (Yamaoka *et al*., 2002a). The importance of adhesins in *H. pylori* colonization in vivo is difficult to determine, as different *H. pylori* strains express a different spectrum of adhesins, and different hosts also show variability in the expression of their adhesin receptors.

1.3.1.6. The *H. pylori* plasticity region virulence factors

Comparison of the genome sequences from two individual strains has shown that approximately 6%-7% of the *H. pylori* genes present in one strain are absent from the other and vice versa (Alm *et al*., 1999). About half of the strain-specific genes are found in a hyper variable region; the plasticity zone. Genes located in such plasticity regions are often associated with increased virulence (Alm and Trust, 1999). In *H. pylori*, the plasticity zone indeed encodes Vir-type ATPases, which are involved in a type IV secretion system which is important in, for example, secretion of the Cag A cytotoxin (Odenbreit *et al*., 2000). Several *H. pylori* genes located in the plasticity zone have been described that are linked to *H. pylori*-related gastro duodenal diseases, such as *H. pylori* Jhp0947 and Jhp0949 are associated with duodenal ulcer disease (de Jonge *et al*., 2004). Both genes are located on the *H. pylori* strain J99 plasticity zone, and are important in *H. pylori*-mediated IL-12 release from monocytes in an in vitro test system (de Jonge *et al*., 2004). The cytokine IL-12 is essential for skewing the immune reaction toward a proinflammatory Th1 response, and is strongly associated with duodenal ulcer formation (Bauditz *et al*., 1999: Trinchieri, 2003). The function of the proteins encoded by *jhp0947* and *jhp0949* is unknown. However, it has been hypothesized that these proteins induce the production of the proinflammatory cytokine IL-12, through the interaction with monocytes. Increased IL-12 levels cause
differentiation of naïve T-cells into activated Th1 cells, leading to an augmented inflammatory response and increased tissue damage (Trinchieri, 2003). An association was found between Jhp0947 and the presence of the cag PAI. Therefore, Jhp0947 was considered a surrogate marker for duodenal ulcer and gastric carcinoma disease (Santos et al., 2003). However, other studies determined that neither Jhp0947 nor Jhp0949 were linked to cag A presence, and thus are independent markers for the development of duodenal ulcers (de Jonge et al., 2004; Salama et al., 2001). The difference in an association of Jhp0947 with cagA might relate to differences between H. pylori isolates from different geographic regions as reported for other virulence factors (Yamaoka et al., 1999; de Jonge et al., 2004). This underlines the notion that the overall effects of H. pylori-factors in disease development are complex and predictions on the clinical outcome of H. pylori infection may be ambiguous. H. pylori Jhp0950, encoding a protein with unknown function is also part of the J99 plasticity zone (Alm and Trust, 1999). The presence of this gene is associated with cagA, cagE, vacA slm1, babA2, hopQ T1 and oipA 'on' (Lehours et al., 2004). The gene cluster comprising Jhp0950, iceA1 and sabA 'on' is significantly more prevalent among H. pylori isolates obtained from patients with a MALT lymphoma than among isolates from patients with gastritis (Lehours et al., 2004). Jhp0950 is found adjacent to Jhp0949 in the H. pylori J99 genome. Jhp0950, unlike Jhp0949, is not associated with an increased risk for duodenal ulcer (Alm et al., 1999). Their associations with disease still support the notion that H. pylori genes located within the plasticity region may be associated with virulence (Alm and Trust, 1999).
Identification of additional strain-specific genes related to pathogenesis has been facilitated by the use of *H. pylori* whole genome microarrays that contain representations of each known *H. pylori* gene. Microarray-based comparisons of genetic content between strains allow each gene within the genome to be sampled simultaneously, and since evolutionary pressures tend to select for the coinheritance of genes involved in common pathways, identification of genes that segregate with known *H. pylori* virulence-related loci may reveal functional relationships that are not evident from sequence data alone. A subset of genes has now been identified using array technologies that covary with the presence of the *cag* island, including two that encode predicted outer membrane proteins: *omp27* and *babA* (Salama et al., 2000). Using a similar microarray analysis, seven genes are identified that were regulated by *HP0164*, which encodes an *H. pylori* sensory histidine kinase (Forsyth et al., 2002) as well as several novel genetic elements that are only present in *H. pylori* *cag* positive strains (Queiroz et al., 2004). *H. pylori* arrays have also been used to identify putative virulence components that may regulate inflammation and injury in vivo. In one study, 25 gastric cancer strains and 71 control *H. pylori* strains (gastritis and no pathology) were phenotyped based on their ability to bind two different classes of epithelial cell receptors (Bjorkholm et al., 2002). From this group, 13 binding and two nonbinding strains were selected for microarray analysis which identified a panel of variable genes, and then each strain was used to challenge germ-free transgenic mice that had previously been shown to develop enhanced pathology in response to *H. pylori* infection. Eighteen variable genes were directly related to the severity of the host response, and the proportion of genes encoding HsdS homologs was significantly higher in this pool compared to the pool of variable
genes that were not related to pathology, suggesting that HsdS homologs may regulate expression of *H. pylori* virulence constituents within the gastric niche (Bjorkholm *et al.*, 2002).

An RNA capture technique been used in conjunction with microarrays to identify *H. pylori* genes that were selectively expressed within gastric tissue. A small subset of *H. pylori* ORFs was consistently present within infected mucosa that was not detected in bacteria grown in vitro, and the majority of these transcripts encoded factors unique to *H. pylori* (Graham *et al.*, 2002). Although many of these in vivo selective RNAs encode hypothetical proteins, one (HP0228) is predicted to encode a membrane protein involved in the transport of sulfate anion (Graham *et al.*, 2002), which is likely to be important for the in vivo survival of *H. pylori*. If these genes are consistently found to be related to distinct disease outcomes, they may represent novel molecular epidemiologic markers, which could identify colonized persons at increased risk for clinical sequelae, who then might be considered for eradication therapy.

1.4. Host responses to *H. pylori*

1.4.1 Host immune evasion by *H. pylori*

If a bacterial species is to persistently colonize its host, it’s most formidable challenge is to evade immune clearance. One mechanism through which *H. pylori* may persist is by limiting the bactericidal effects of proinflammatory molecules, such as nitric oxide (Gobert *et al.*, 2001). *H. pylori* possess a gene, rocF that encodes a functional arginase which siphons L-arginine away from a competing host enzyme, inducible nitric oxide synthase (iNOS). This, in turn, limits the production of iNOS-generated nitric oxide by limiting the availability of the L-
arginine substrate (Gobert et al., 2001). Another level of host defense that may be circumvented by H. pylori is innate immunity. Toll-like receptors (TLRs) are an evolutionarily conserved family of eukaryotic receptors that function in innate immunity via recognition of invariant regions in bacterial molecules termed pathogen- or microbe-associated molecular patterns. Eleven different TLRs have been identified in mammals, and although the bacterial ligands for TLRs are distinct, signaling pathways utilized by these receptors all appear to eventuate in NF-κB activation and proinflammatory gene expression. H. pylori have evolved strategies to avoid global activation of this system. For example, TLR4 recognizes bacterial LPS, yet H. pylori LPS is relatively anergic compared with that of other enteric bacteria such as Escherichia coli (Blaser and Atherton, 2004; Perez-Perez et al., 1995). In contrast to proinflammatory TLR5-activating flagellins expressed and secreted by mucosal pathogens such as Salmonella, H. pylori flagellin is not secreted and is noninflammatory (Gewirtz et al., 2004; Lee et al., 2003). H. pylori infection is associated with increased gastric mucosal levels of IL-10 (Peek et al., 1995), an anti-inflammatory peptide that inhibits secretion of proinflammatory chemokines from macrophages and neutrophils.

H. pylori can facilitate its own persistence by varying the antigenic repertoire of surface-exposed proteins, such as Cag Y (Aras et al., 2003), and by actively suppressing the adaptive immune response of the host (Boncristiano et al., 2003; Gebert et al., 2003; Sundrud et al., 2004). H. pylori can also survive and replicate within epithelial cells and macrophages (Amieva et al., 2002; Zheng and Jones, 2003), thereby evading host clearance. However, despite these multiple strategies for persistence, substantial immune activation still occurs as evidenced by epithelial proinflammatory cytokine release, infiltration of the gastric mucosa by
inflammatory cells, and cellular and humoral recognition of *H. pylori* antigens (Blaser and Atherton, 2004).

1.4.2. Humoral responses to *H. pylori*

*H. pylori* colonization induces an exuberant systemic and mucosal humoral response directed at multiple antigens; however, antibody production does not result in eradication, even though this organism is susceptible in vitro to antibody-dependent complement-mediated phagocytosis and killing. This ineffective humoral response may not only contribute to persistence but also to pathogenesis. For example, a potential consequence of *H. pylori* Lewis antigen expression is induction of a humoral response that enhances gastric inflammation and injury (Appelmelk *et al.*, 2000). *H. pylori* infection in mice induces anti-Lewis*α* antibodies with an affinity for parietal cells, and immunization of mice leads to the development of anti-Lewis*α/*γ antibodies that cross-react with host Lewis*α/*γ antigens present on H+,K+-ATPase located in parietal cell canaliculi (Appelmelk *et al.*, 1996). In humans, *H. pylori* infection similarly induces autoantibodies directed against the canaliculi of gastric parietal cells (Negrini *et al.*, 1991). IgM antibodies generated by immortalized B cells obtained from *H. pylori*-colonized gastric mucosa also recognize gastric epithelium (Vollmers *et al.*, 1994). These findings indicate that the humoral response to *H. pylori* may initiate and/or maintain mucosal inflammation and epithelial cell injury.

1.5. Molecular techniques for comparative genomic characterization

1.5.1 Suppressive Subtractive Hybridization.

Suppression Subtractive Hybridization (SSH) was initially reported in 1996 [Diathchenko *et al.*, 1996] and was first applied to bacteria in a study of
*Helicobacter pylori* in 1998 [Akopyants et al., 1998]. The kit for SSH is now available commercially (Clontech PCR-Select Bacterial Genome Subtraction Kit, BD Clontech UK, Hampshire). The kit has been applied to several different bacterial pathogens including *Aeromonas hydrophila* (Zhang et al., 2000), *Burkholderia pseudomallei* (Reckseidler et al., 2001), *Escherichia coli*, *Salmonella* (Bogush et al., 1999; Janke et al., 2001) and *Klebsiella pneumoniae* (Lai et al., 2000). Recently SSH used for identification of markers for *H. pylori* strains isolated from children with peptic ulcer disease (Oleastro et al., 2006). Briefly, tester strain DNA is digested with a restriction endonuclease (such as Rsal) and separated into two portions, each of which is subjected to a ligation reaction to attach a different adaptor sequence to the 5' ends. The two portions are then separately hybridised to driver strain DNA (in excess). Any sequences that hybridise with the driver DNA should be 'mopped up', leaving only tester specific single-stranded sequences. When the two portions are eventually mixed and hybridised together, allowing homologous single-stranded DNAs to hybridise, only those sequences unique to the tester strain will have different adaptors present on each strand. These sequences are detected by PCR amplification with primers designed to bind to adaptor sequences. The key to the success of this strategy is that sequences containing the same adaptor sequence at both ends are unable to amplify because of a suppression effect whereby a secondary structure forms to prevent primer annealing (Clontech PCR-Select™ Bacterial Genome Subtraction Kit). Consequently, only those sequences now carrying both adaptors (one at each end) will amplify. These PCR products are cloned into a suitable vector to produce a subtracted DNA library. Although the procedure is not entirely effective, > 50% of clones should be tester-specific. This can be tested by labelling clones and probing the tester and driver strains.
1.5.2. DNA Microarray

A Microarray is a set of miniaturized chemical reaction areas that may also be used to test DNA fragments, antibodies, or proteins, by using a chip having immobilised target and hybridising them with probed sample. The color from the chip after hybridization is then scanned and the data is analysed by software to find the expression array. Microarray technology was first introduced by Schena and Colleagues in 1995 (Schena et al., 1995). The purpose of a microarray is to examine expression of multiple genes simultaneously in response to some biological perturbation. More generally, a microarray serves to interrogate the concentration of molecules in a complex mixture and thus can serve as a powerful analytical tool for many kinds of experiments. As this technology is a tremendously powerful method for simultaneously monitoring the expression of thousands of genes in a single experiment, it has become widely applied tool in biological and medical research, including the discovery of drug-sensitive genes (Ivanov et al., 2005), development of biomarkers for classification of disease subgroups and monitoring of disease progression (Kittleson and Hare, 2005; Pusztai and Hess, 2004; Wadlow and Ramaswamy, 2005). There are two major types of microarray platforms that are commonly used, cDNA arrays and Oligonucleotide arrays. cDNA (200-5000 bases long) are immobilized onto a solid surface such as a glass slide or nylon membrane. Probes for cDNA arrays are usually products of polymerase chain reaction (PCR) generated from cDNA libraries or clone collections. Using this technique, arrays consisting of more than 30000 cDNA can be fitted onto the surface of a conventional microscope slide. The use of cDNA probes increases the sensitivity of the array because they are typically much longer than oligonucleotide probes. However, sensitivity comes with a loss of specificity due to cross-hybridization. For oligonucleotide arrays, a typical array is Affymetrix’s GeneChip,
where the short oligonucleotide (20-25 mers) probes are synthesized in situ using a photolithographic method. The process of expression analysis can be divided into several stages: 1) Experimental design; 2) sample collection and RNA extraction; 3) Sample labelling; 4) Hybridization, Detection and Scanning; 5) Identification of differentially expressed genes; 6) Cluster analysis, pathway identification and functional classification; 7) Validation and data.

1.5.3. Proteomics

Proteome was defined for the first time in 1995 as the "total protein complement of a genome" (Wasinger et al., 1995) or, in the case of a pluricellular organism, as the protein complement of a tissue. In other words, proteomics is the study of the properties of proteins (expression level, post-translational modifications, and interactions) on a large scale to obtain an integrated view of normal or pathological cellular processes or interactions at the protein level.

Current research in proteomics requires first that proteins be resolved, sometimes on a massive scale. Protein separation can be performed using two-dimensional gel electrophoresis, which usually separates proteins first by isoelectric point and then by molecular weight. Protein spots in a gel can be visualized using a variety of chemical stains or fluorescent markers. Proteins can often be quantified by the intensity of their stain. Once proteins are separated and quantified, they are identified, usually by in gel digestion and subsequent mass spectrometry. For the in-gel digestion, individual spots are cut out of the gel and cleaved into peptides with proteolytic enzyme. These peptides are used for the identification of the protein by peptide mass fingerprinting or de novo sequencing (Hecker et al., 2008)
The peptide mass fingerprint relies on the specific pattern of peptide signals for a given protein in mass spectrometry, most often MALDI-TOF mass spectrometry. The pattern obtained in mass spectrometry is compared with database entries for the identification of the protein. Understanding the proteome, the structure and function of each protein and the complexities of protein-protein interactions will be critical for developing the most effective diagnostic techniques and disease treatments in the future. An interesting use of proteomics is using specific protein biomarkers to diagnose disease. A number of techniques allow to test for proteins produced during a particular disease, which helps to diagnose the disease quickly. Techniques include western blot, immunohistochemical staining, enzyme linked immunosorbent assay (ELISA) or mass spectrometry.

Pathogenic bacteria are suitable for proteomic comparisons with the aim of elucidating proteins with vaccine and diagnostic applications, as well as determining novel targets for drug design and the effects of these drugs on cellular physiology (Ahn and Wang 2008).
1.6. AIM S AND OBJECTIVES

1.6.1 General Objectives:-

The overall objective of the thesis is to determine the genetic basis by which *H. pylori* cause's gastro duodenal diseases. In addition it aims to identify and characterize biomarker of *H. pylori* isolates from Irish adult patients with gastro duodenal diseases.

1.6.2. Specific Objectives:-

1) To characterise the Pathogenicity Island, vacA subtype, in *H. pylori* strains and to investigate their relations to *H. pylori*-associated gastro duodenal diseases.

2) To evaluate the effect of other virulence factors such as *babA2*, *iceA*, and *oipA*, in such groups of patients.

3) To investigate the importance of the genes in the Plasticity region in the pathogenesis of *H.pylori*, and its correlation with the gastro duodenal diseases.

4) To test and search for disease specific protein marker candidates of *H.pylori* strains isolated from patients with gastro duodenal diseases.
Table 1.1. Landmark research on *Helicobacter pylori*

<table>
<thead>
<tr>
<th>Years</th>
<th>Event</th>
</tr>
</thead>
<tbody>
<tr>
<td>1875</td>
<td>Bottcher and Letulle found spiral bacteria in animal stomachs.</td>
</tr>
<tr>
<td>1905</td>
<td>Reigel suggested that ulcers are caused by excess acid.</td>
</tr>
<tr>
<td>1906</td>
<td>Krienitz found spiral bacteria in human cancerous stomachs.</td>
</tr>
<tr>
<td>1910</td>
<td>Schwartz published the excess acid theory of the ulcer “No acid, No ulcer”.</td>
</tr>
<tr>
<td>1915</td>
<td>Antacids were first recommended for the treatment of peptic ulcer disease</td>
</tr>
<tr>
<td>1924</td>
<td>Luck and Seth discovered urease in the human stomach.</td>
</tr>
<tr>
<td>1951</td>
<td>Allende first described the treatment of gastric ulcers with penicillin.</td>
</tr>
<tr>
<td>1982</td>
<td>April 8-first successful culturing of <em>H. pylori</em>.</td>
</tr>
<tr>
<td>1983</td>
<td>Marshall and Warren identified Campylobacter like bacteria associated with gastritis-beginning of modern era</td>
</tr>
<tr>
<td>1990</td>
<td>Rauws and Tytgat described cure of duodenal ulcer by eradication <em>H. pylori</em> using a triple-therapy regimen.</td>
</tr>
<tr>
<td>1990</td>
<td>World Congress of Gastroenterology recommended eradicating <em>H. pylori</em> in order to cure duodenal ulcer.</td>
</tr>
<tr>
<td>1992</td>
<td>Covacci et al sequenced the <em>cagA</em> gene. This was the first description of a virulence factor of <em>H. pylori</em> determined by molecular technique.</td>
</tr>
<tr>
<td>1993</td>
<td>Bazzoli et al started the proton-pump inhibitor-based triple therapy.</td>
</tr>
<tr>
<td>1994</td>
<td>The World Health Organization’s International Agency for Research on Cancer declared <em>H. pylori</em> as a class 1 carcinogen.</td>
</tr>
<tr>
<td>1994</td>
<td>Michetti et al. tested a urease-based vaccine in a mouse model.</td>
</tr>
<tr>
<td>1997</td>
<td>Tomb et al. sequenced the entire <em>H. pylori</em> genome.</td>
</tr>
<tr>
<td>1998</td>
<td>Ilver et al. identified BabA.</td>
</tr>
<tr>
<td>1999</td>
<td>Michetti et al. tested urease vaccine in humans (although it failed).</td>
</tr>
<tr>
<td>2005</td>
<td>Marshall and Warren were awarded the Nobel Prize in Physiology or Medicine for their work on relationship of <em>H. pylori</em> and peptic ulcer disease</td>
</tr>
<tr>
<td>2007</td>
<td>Rhead et al. identified (i) region a 3rd polymorphic determinant of <em>vacA</em> gene.</td>
</tr>
<tr>
<td>2009</td>
<td>Baltrus et al. sequenced the 4th <em>H. pylori</em> Strain G27</td>
</tr>
</tbody>
</table>
Table 1.2. Determinants important in *H. pylori*-associated disease outcome

<table>
<thead>
<tr>
<th>Determinant</th>
<th>Disease association</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Host</strong></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>Early age of acquisition</td>
</tr>
<tr>
<td>Inflammation</td>
<td>Th 1 response</td>
</tr>
<tr>
<td></td>
<td>IL-1β/IL-1RA/TNF-α/IL-10 Polymorphism</td>
</tr>
<tr>
<td>Acid secretion</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td>Low</td>
</tr>
<tr>
<td><strong>Environment</strong></td>
<td></td>
</tr>
<tr>
<td>Smoking</td>
<td></td>
</tr>
<tr>
<td>Diet</td>
<td>High salt/carbohydrates?</td>
</tr>
<tr>
<td></td>
<td>Low vitamin C7/B-carotene?</td>
</tr>
<tr>
<td>Medication</td>
<td>NSAID</td>
</tr>
<tr>
<td><strong>H. pylori</strong></td>
<td></td>
</tr>
<tr>
<td>CagA</td>
<td>cagA positive</td>
</tr>
<tr>
<td>VacA</td>
<td>vacAs1 positive</td>
</tr>
<tr>
<td>iceA</td>
<td>iceA1 or iceA2 positive</td>
</tr>
<tr>
<td>BabA</td>
<td>BabA2 positive</td>
</tr>
<tr>
<td>OipA</td>
<td>oipA ‘on’</td>
</tr>
<tr>
<td>Jhp0947</td>
<td>Jhp0947 positive</td>
</tr>
<tr>
<td>Jhp0949</td>
<td>Jhp0949 positive</td>
</tr>
<tr>
<td>Jhp1462</td>
<td>Jhp1462 positive</td>
</tr>
</tbody>
</table>
Figure: 1.2. Proposed Interaction between Host, Environment and H. pylori Infection in the development of Gastroduodenal Diseases

- **Environment factors** e.g., smoking, malnutrition, high salt intake, vitamin deficiency
- **Host factors** e.g., IL-1β polymorphism

- **H. pylori infection**
  - High acid output
    - Gastrin
    - **Antrum-predominant gastritis**
      - Increased duodenal acid load
      - **H. pylori colonization of gastric metaplasia in duodenum**
      - Weakened duodenal mucosal protection by intense inflammation
      - **Duodenal ulcer**
  
  - **Low acid output**
    - **Diffuse**
    - **Bacterial virulence factors**
      - **Atrophy**
      - **Gastric ulcer and Cancer**

- **Increased H. pylori colonization in corpus**
CHAPTER 2
GENERAL MATERIALS AND METHODS
2.1. *H. pylori* strains

Two hundred and twenty six patients who were infected with *H. pylori* were included in the study. Eighty-six (86) *H. pylori* DNA (Chapter 3) samples were extracted and obtained from formalin fixed paraffin embedded tissue (FFPE) from archival paraffin-embedded tissue obtained from the Department of Pathology, at Adelaide and Meath Hospital, incorporating the National Childrens Hospital (AMNCH), Tallaght, Dublin. Another one hundred and forty (140) clinical *H. pylori* isolates. (Chapters 4 and 5) were obtained from gastric biopsy specimens from patients undergoing routine upper gastrointestinal endoscopy at (AMNCH).

Gastric biopsies were obtained with informed consent from all patients under standard protocols approved by the Joint Research Ethics committee at (AMNCH).

2.1.1. Detection and Primary Isolation of *H. pylori* from Gastric Biopsies

Five gastric biopsies were obtained from the antrum and corpus of each patient. Patients with a history of antibiotic use within the previous month and those taking proton pump inhibitors (PPI's) within the previous two weeks were excluded. At endoscopy, five gastric biopsy specimens were obtained for *H. pylori* diagnosis. The determination of the *H. pylori* status involved three tests, Histology, Rapid urease Test (CLO™), and Culture. Two of these tests had to be positive to deem the patient to be infected. The histological determination of *H. pylori* infection was based on gastric mucosal biopsy tissue taken at the time of endoscopy. Where *H. pylori* was observed (by a pathologist) in Hematoxylin and Eosin stained gastric mucosal biopsy tissue sections, results were expressed semi-quantitatively according to the updated Sydney System (Dixon *et al* 1996). Gastric biopsies for culture were placed in a transport medium (nutrient broth), and kept
at -70° for 24 hours. When the result of the rapid urease test was positive, such biopsies were inoculated, mucosal side downwards, on to the surface of two fresh blood agar plates consisting of Columbia agar base (Oxoid, UK) supplemented with DENT-supplement (Oxoid, UK) and 7% horse blood (Jones, 1984). Plates were subsequently incubated at 37°C for 4-7 days under microaerobic atmosphere using a CO₂ Generation Kit (Oxoid, UK). The presence of *H. pylori* from gastric biopsies was confirmed by subjecting re-streaked presumptive *H. pylori* colonies from primary isolation plates were Gram stained and urease activity confirmed (Christensen’s agar slopes containing 40% urea; Oxoid Ltd., Basingstoke, UK).

2.1.2. Gold standard definition of *H. pylori* diagnosis.

A patient was classified as being *H. pylori* positive on at least two positive results of rapid urease test, histology and culture. The *H. pylori* - related gastroduodenal disease was classified on the basis of the endoscopic findings or the histological findings.

2.1.3. Culture of *H. pylori* in liquid medium

To obtain a sufficient amount of *H. pylori* liquid cultured *H. pylori* bacterial cells were resuspended in the medium till solution had an optical density of 0.1-0.2. The liquid medium with bacteria was poured on to cell culture flasks and incubated in a microaerophilic incubator at 37°C by shaking at 120 r p m. After 24 hours the optical density of the bacterial suspension reached 0.7-1 and the bacterial cells were harvested.

2.1.4. Storage and recovery of *H. pylori*

*H. pylori* isolates were subcultured in duplicate on to blood agar and incubated under microaerophilic condition for 3 days. Using a sterile loop, the bacterial
colonies were harvested and placed into a sterile 1.5 ml freezer vial (Sarstedt, Germany) containing 1 ml of storage medium consisting of BHI (Oxoid, UK), 20% (v/v) glycerol and 10% (v/v) filtered horse serum (Oxoid, UK). Storage vials were briefly vortexed and frozen immediately at -80°C. When required, storage vials were removed from the freezer and allowed to defrost at room temperature. To aid bacterial recovery, 10 µl of the suspension was removed with a sterile loop and four 2-cm² areas of two blood agar plates were inoculated. Plates were incubated for 3-5 days and subcultured twice before use.

2.2 Routine Molecular Techniques

Phenol-chloroform extraction of DNA, and clean up of PCR reactions, were all carried out according to standard protocols (Ausubel et al., 1987, Sambrook et al., 1989) with manufacturer’s instructions. Commercially purchased kits e.g. protein determination, PCR-product purification were also used according to the manufacturer’s specifications.

2.3. DNA isolation and analysis

2.3.1. Extraction of *H. pylori* genomic DNA

Bacterial genomic DNA was purified according to the method described by Ausubel et al. (1987) with some modifications. Bacterial growth was harvested from 3 day-old blood agar plates and resuspended in 1 ml of sterile distilled water or Phosphate Buffered Saline (PBS). The bacterial cells were pelleted by centrifugation at (~15,000 rpm) for 5 min using a bench-top micro centrifuge. This procedure was repeated and the pellet was resuspended in 567 µl Tris EDTA buffer (TE), to which 30 µl of 10% Sodium Dodecyl Sulphate (SDS) (w/v) and 3 µl of
proteinase K (20mg/ml) were added. The suspension was incubated at 37°C in a water bath. After 1 h, 100 μl of 5 M NaCl was added and the suspension was mixed thoroughly. Then 80 μl of cetyltrimethyl ammonium bromide (CTAB)-NaCl solution [10% (w/v) CTAB in 0.7 M NaCl] was added and the solution was incubated for 10 min at 65°C in a water bath. An equal volume of chloroform: isoamyl alcohol (24:1) was then added to the solution, mixed thoroughly and centrifuged at (~15,000 rpm) for 5 min. The upper aqueous layer containing the DNA was collected and an equal volume of phenol: chloroform: isoamyl alcohol (24:24:1) was added. The solution was thoroughly mixed, centrifuged and the aqueous layer was collected as before. Isopropanol (0.6 vol) was added and the micro centrifuge tube was gently inverted to precipitate the DNA. The DNA was pelleted by centrifugation for 30 min at 4°C. The pellet was washed twice with 1 ml of 70% (v/v) ethanol and then air-dried for 20 min. The genomic DNA was resuspended in 50-100 μL of Tris EDTA buffer.

2.3.2. DNA extraction from formalin fixed paraffin embedded (FFPE) tissue

DNA from *H. pylori* in patients with Intestinal Metaplasia (IM) or Chronic Gastritis (CG) included in the study was extracted from archival gastric mucosal biopsy specimens. DNA Isolation Kit for Mouse Tail Tissue (Gentra®, USA) was used. Briefly, 5 x 0.5μm sections of FFPE tissue were cut on a microtome (Leica, Germany) and placed in 1.5 ml micro centrifuge tubes to which 300μl Xylene (Scarlaau, Spain) was added. The tubes were then centrifuged at full speed (14000rpm) for 3 min and the Xylene was then discarded. This procedure was repeated twice. 300μl ethanol (Aldrich, USA) was then added to the tissue, the tubes were vortexed for 5 min at room temperature, spun at 14,000 rpm for 3 min, and the ethanol discarded. This procedure was then repeated. Cell lysis
solution (300μl, Gentra®, USA) was then added to the tissue and the contents of
the tube thoroughly mixed. 1.5 μl Proteinase K (20 mg/ml, Gentra®, USA) was
added to the tissue, and each tube was inverted many times and left to incubate
at 55°C for 24 hours. Then, a further 1.5 μl Proteinase K was added and the tubes
returned to incubate at 55°C for a further 24 hours, after which the process was
repeated. The supernatant (containing the DNA) in each tube was then poured off
into a 1.5 ml micro centrifuge tube containing 300 μl Isopropanol (Sigma-Aldrich,
Germany) and 0.5 μl Glycogen (20mg/ml, Roche Diagnostics, Germany). The tubes
were then inverted 50 times and centrifuged at 14,000 rpm for 5 min. Following
centrifugation the supernatant was poured onto clean absorbent paper, 300 μl of
70% ethanol was added and the tubes inverted. The tubes were then centrifuged
again at 14,000 rpm for 1 min and the ethanol poured off. Each tube was then left
open to air dry at room temperature for 15 min. 20 μl DNA Hydration Solution
(Gentra®, USA) was then added and the DNA rehydrated by incubating at 65°C for
1 hour. DNA was then stored at -20°C.

To determine the concentration, quality, and relative purity of the DNA samples,
the ultraviolet spectrophotometric method/or agarose gel electrophoresis method
were used as outlined by Sambrook et al. (1989). Briefly, for spectrophotometrical
quantification of DNA samples, 5 μl of DNA sample was diluted with distilled
water. The sample then measured at 260 nm and 280 nm wavelength. The \( A_{260} \)
at 1 was equivalent to 50μg ml\(^{-1}\)-DNA. For determining the purity, the \( A_{260} / A_{280} \) ratio
greater than 1.8 for DNA, which means a pure DNA sample. For determining the
DNA concentration with agarose gel analysis, a DNA sample was run on 1%
agarose gel in parallel with a series of DNA standards of known amounts (e.g.0,
2.5, 5, 10, 20, 30, 40, 50 μg ml\(^{-1}\)). The concentration of the DNA samples was
then estimated by comparing the intensity of the sample DNA band with the DNA standards.

2.3.3. Agarose gel electrophoresis

DNA was separated on 0.8 to 2% agarose gels in 1X Tris Acetate EDTA (TAE) buffer or 0.5XTBE buffer. Gel loading buffer was added to all DNA samples at a ratio of 1:5. Electrophoresis was performed using Bio-Rad horizontal electrophoresis tanks. Gels were prepared using type II medium agarose (Sigma) and freshly diluted Tris Borate Buffer (0.5).

Electrophoresis was preformed under the following conditions:

<table>
<thead>
<tr>
<th>DNA samples</th>
<th>Agarose concentration</th>
<th>Voltage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% (w/v)</td>
<td>(v)</td>
</tr>
<tr>
<td>PCR product &lt; 800bp</td>
<td>2</td>
<td>75</td>
</tr>
<tr>
<td>PCR product &gt; 800bp</td>
<td>1.5</td>
<td>75</td>
</tr>
<tr>
<td>Chromosomal DNA</td>
<td>0.8</td>
<td>75</td>
</tr>
</tbody>
</table>

Gels were pre-stained by adding ethidium bromide (10mg/ml) to the cooled molten agarose to a final concentration of 0.5mg/ml. Electrophoresed DNA was visualised by exposure of the gel to ultra-violet light.

2.3.4. Polymerase Chain Reaction (PCR)

PCR was performed on chromosomal DNA isolated from three day old agar cultures of H. pylori. The sequences of the oligonucleotides used as primers and their corresponding PCR product sizes are listed in (Table 4.4; Table 5.2). The PCR
amplification was carried out in a final volume of 50 µl containing 1 µl DNA template (20–100 ng µl), 1.5mM MgCl₂, 0.4–0.8 each primer, 1.5 U Taq polymerase (Promega Corp., Madison, USA), 200 µM each of dATP, dCTP, dGTP, dTTP, and 1X PCR buffer. The thermal cycling parameters were 1 cycle of 94°C followed by 30-35 cycles of denaturing at 94°C (0.5-1 min), annealing at appropriate temperature (0.5-1) and extension at 72°C (0.5-1 min). A final extension at 72°C for 10 min was included in the amplification. Synthesis of the primers was provided by the service from MWG Biotech Ltd, Germany. PCR was performed at least twice for each sample.

2.3.5. Nested polymerase chain reaction (Nested-PCR)

Nested PCR for detection and genotyping of the *H. pylori cagA, iceA* subtype, was performed using the primers shown in (Table3.1). The selected primers were based on the sequences of the *cagA, iceA* genes published in the Genbank database of National Center Biotechnology Information (NCBI). All the amplifications were carried out in a thermal cycler (MJ Research PTC-2000-USA). The first round reaction was performed in 25 µl final volume containing 0.25 mM of each dNTPs (Promega-USA), 3.0 mM MgCl₂, 1.25 U Taq polymerase (Promega-USA), and 10–20 ng of template DNA and 10 pmol of each of the first round primers. The initial denaturation was carried out for 5 min at 95°C and cycling was performed as follows 94 °C for 30 s, 56 °C for 30 s, 72°C for 30 s for 30 cycles. The final cycle included an extension for 7 min at 72°C to ensure full extension of the product. After the first round, 1 microliter of the reaction mixture was transferred to the second round reaction mixture containing 10 pmol of each inner primers and the same buffer as in the first round, except that for the second PCR only 25 amplification cycles were used. DNA from the *H. pylori* reference
strain ATCC 26695 acted as positive control and a tube containing distilled water in place of DNA was used as negative reagent control with each batch of amplifications. Extensive care was taken to prevent false positive results due to contamination. To identify the amplified products, five μl of the first and second round PCR products were analyzed by electrophoresis on 1.5% agarose gels (Bio Rad), containing 0.5 μg of ethidium bromide per ml for visualization. Single-step PCR was carried out for ureC as an internal control (house-keeping gene), a vacA subtype gene.

2.3.6. Evaluation of the functional status of oipA gene

The functional status of the oipA (HP638/jhp581) gene is regulated by a slipped strand repair based on the number of CT dinucleotide repeats in the signal coding region. The signal sequences of the oipA gene including the CT repeats were amplified by PCR with the primer designations as F1-Hp0637-jhp580 CCCCACAAGCGCTTAACAG (sense), and F1-Hp0638-jhp581 GAGAGTGCCTAAACCCTATAATCC (antisense). These primers were also used for sequencing. The PCR amplification for oipA was carried out in a 25 μl volume containing 2.5μl of 10 X PCR buffer (Promega), 1.5 mM MgCl₂ (Promega), 200 μM each deoxynucleoside triphosphates (dNTPs), 2 U of Taq DNA polymerase, 1 μM of forward and reverse primers, and 20 ng of H. pylori DNA. After 4 min of denaturation at 94°C, the reaction mixture was amplified for 35 cycles as follows, 30 s at 94 °C, 30 s of annealing at 60°C, and 30s at 72°C. After the last cycle, extension was continued for another 7 min. The PCR products were purified using a PCR Purification Kit (Qiagen) following the manufacturer’s instructions. Determination of the nucleotide sequences was provided by the service from
(EurofinsMWG Operon Ltd, Germany). Sequence analyses were then carried out using the appropriate programs available on the Internet (http://www.ebi.ac.uk or http://www.ncbi.nih.org).

2.4 Fingerprinting of *H. pylori* isolates.

2.4.1. Random amplified polymorphic DNA-PCR (RAPD-PCR) fingerprinting

*H. pylori* isolates were fingerprinted by RAPD-PCR (Akopyanz *et al.*, 1992) using different RAPD primers. RAPD-PCR reactions took place in a final volume of 25μl that included 10 X reaction buffer, 3 mM MgCl₂, 10 pmol each primer, 0.4 mM each of dATP, dCTP, dGTP, dTTP, 0.5 units of Taq polymerase (Takara, UK) and 1 μl genomic DNA as template (50-100ng/μl). The cycling parameters routinely employed for RAPD primers of 10 bases (primers 1254, 1283), were as follows: 4 cycles of 94 °C for 5 min, 36 °C for 5 min, 72 °C for 5 min (low stringency synthesis steps) followed immediately by amplification at higher stringency for 30 cycles of 94°C for 1 min, 36 °C for 1 min, 72 °C for 2 min. A final 10 min extension at 72 °C was carried out to finish incomplete elongation of products. RAPD-PCR products were electrophoresed through 2 % (w/v) agarose gels containing ethidium bromide (0.5μg/ml) and fingerprints visualised by exposure of the gel to ultraviolet light.

2.5. Proteomic Analysis

2.5.1 Characterization of *H. pylori* clinical isolates.

*H. pylori* strains were isolated from biopsy material of gastric antral mucosa, which obtained from three groups of patients with gastro duodenal diseases.

2.5.2. Sample preparation

*H. pylori* cells were harvested for cell lysate preparation from 48-72-h lawned plate cultures using sterile transfer pipettes into 1.5 ml of distilled water, and
pelleted by centrifugation at 14,000 rpm for 5 min, then suspended in distilled water. Following three cycles of sonication with 10 amplitude microns (Soniprep, 150-Sanyo/Japan), for 30 seconds. The crude extract was centrifuged at high speed for 4 min at 4°C to remove unbroken cells and debris. The supernatant was stored at -20°C, or used immediately for the colorimetric detection and quantitation of total protein in each sample/strain, using Pierce®, BCA Protein Assay Kit (Thermo-scientific-USA), following the instructions of the manufacture. A specific amount of protein from each sample (150-250 µg), obtained from the supernatant of the sample protein-acetone cold, and four volume of acetone to one volume of the sample protein solution, kept at -20°C overnight for quantitative recovery, then spin in minifuge tube (High speed) for 5 min at room temperature. Aspirate the supernatant and retain pellet, and each tube was then left open to air dry at room temperature for 15 min.

2.5.3. Two-dimensional electrophoretic analysis of \textit{H. pylori} proteins.

The first Two-electrophoresis dimension was performed using 13-cm pH3-10 Immobiline Dry strip with sample in gel rehydration and the Amersham Ettan™ IPGphor II™ isoelectric focusing system (GE Healthcare-UK). Approximately 400 µg protein was added to 450 µl of Rehydration Buffer containing urea (8 mol/L), 0.5g CHAPS (2%, w/v) (3-(cholamidopropyl) dimethyl-amino)-1-propanesulfonate), IPG buffer (0.5%v/v) and bromophenol blue (0.002%, v/v). The solubilise sample then centrifuge at 14,000rpm for 4 min in a minifuge prior to the sample loading for IEF step to remove any insoluble material. Overlay with cover fluid and perform isoelectric focusing on 13 cm IPG strips for approximately 18 hours. The voltage was linearly increased from 60V during the first 14 hours and then stabilized for one hour at 500 V, 1000 V, 8000 V respectively (total, 16,600 V/18 h). Then the
Immobilized pH Gradient remove from cassette and either freeze at -20°C for storage or proceed to equilibrate the strip in Equilibration buffer (50Mm Tris.HCl (pH8.8), 6M urea, 30%(v/v) glycerol, 2% (w/v)SDS and bromophenol blue(0.002%, v/v), dithioerythritol (1g/L), and then iodoacetamide(2.5g/L). Then electrophoresis in the second dimension was performed on a 12-15% polyacrylamide linear gradient gel at a constant current of 25mA. Subsequently, gels were fixed and stained for overnight in a 0.25% Coomassie Brilliant Blue [250 mg Coomassie brilliant blue, 90 ml methanol: H₂O (1:1 V/V), 10 ml glacial acetic acid]. Excess dye was removed from the gel by soaking in destain solution (methanol-acetic acid solution and H₂O (3:1:6 V/V)).

2.5.4. Sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

The discontinuous SDS system of Laemmli (1970) was utilized for SDS-PAGE employing the Bio-Rad “Mini PROTEAN II” casting plate and gel tank. Gels were electrophoresed at a constant voltage of 250 V for 90 min. Polypeptides were resolved through separating gels consisting of 12% (w/v) polyacrylamide (made from a 30% stock of acrylamide mix [29% (w/v) acrylamide, 1% (w/v) N,N'-methylenebisacrylamide]), 375 mM Tris-HCl (pH8.8), 0.1%(w/v) SDS, 0.1%(w/v) ammonium presulphate and 0.04%(v/v) N,N',N',N'-tetramethylethlenediamine (TEMED). Stacking gels, consisting of 5% (w/v) polyacrylamide, 125 mM Tris-HCl (pH8.8), 0.1% (w/v) SDS, 0.1% (w/v) ammonium presulphate and 0.001% (v/v) TEMED were also employed in the electrophoresis of H. pylori cell lysates. The bacterial suspension was denatured in sample buffer for SDS-PAGE. All samples were heated at 100°C for 5 min, cooled to RT, and subjected to electrophoresis (30mg of protein loaded in each well) with a constant voltage of 250 V until the dye front reached the bottom of the gel.
2.5.5 Western blotting

Proteins were electrotransferred from gels to nitocellulose sheets by semidry blotting apparatus (Atto). First the gels were washed briefly in transfer buffer containing 50 mmol/L Tris, 39 mmol/L glycine, 1 mmol/L SDS, 200 mL/L methanol. Three sheets of blotting paper already soaked in transfer buffer were placed in the semidry blotting apparatus, then the gel was placed on the top of a PVDE membrane (cutting the same size as gel) and overlay with three sheets of blotting paper. The electrotransfer time was 1 hour with a current/area of 0.9 mA/cm². The membranes were washed in phosphate buffered saline (PBS), blocked for 1 hour at RT with gentle shaking by incubating in PBS containing 5% Marvel. The membranes were incubated for 2 h at RT with human pooled sera from *H. pylori* positive or negative patients at a dilution of 1:700. Immunodetection of IgG was revealed with secondary antibody (goat anti-human horseradish peroxidase conjugated immunoglobulin IgG (Dako-UK) at a dilution of 1:1000 (by volume), for 1 hour at RT. Before and after each step, the membranes were washed extensively 6 times for 10 min in PBS with 0.05% of Tween-20. To visualise the western blot, enhanced chemiluminescence was used for the final detection, first the blot was washed in PBS containing Tween-20, then 200 ml of enhanced chemiluminescence buffer (ECL buffer) [50 Mm Tris(pH8.8), Luminol (14mg), and iodophenol (4 mg dissolved in 500 μl Dimethyl sulfoxide, with 20 μl of hydrogen peroxide(30% v/v))] was added to the western blot, then mixed gently for 1 min and the membrane placed between two sheets of acetate paper and exposed to the X-ray film for various periods of time.
2.5.6 Serum samples

Human sera were obtained from *H. pylori* positive patients affected by different gastric diseases, including duodenal ulcer, intestinal metaplasia, and chronic gastritis, as a negative control a pool of sera was used from three volunteers without gastric disorders and negative for *H. pylori* infection. Patients were considered positive to *H. pylori* infection when both the CLO and histological tests were positive.

2.6 Statistical analysis

Statistical analysis was performed using SPSS V/0 statistical analysis software. The chi-square test or the Fisher's exact test was used where applicable. A *p* value lower than 0.05 was considered as a statistically significant difference.
Chapter 3
DETECTION OF VIRULENCE MARKERS IN *HELIcobacter pylori* FROM ARCHIVAL GASTRIC BIOPSIES
3.1. Introduction

*H. pylori* is a highly prevalent bacterial pathogen that persistently colonizes the mucosa of the human stomach (Ernst and Gold, 2000). *H. pylori* infection invariably results in chronic gastritis and subsequently can result in diseases, such as peptic ulcer disease, gastric carcinoma, and MALT lymphoma (Spencer and Wotherspoon 1997; Graham, 2000; Gatti et al., 2005). Genotyping alterations of *H. pylori* are thought to be responsible for the various clinical manifestations of infection that ranging from asymptomatic chronic gastritis to gastric carcinoma and MALT lymphoma. There are two phenotypically distinct *H. pylori* groups: type one *H. pylori*, which express the cytotoxin-associated gene antigen (cagA), and the vacuolating cytotoxin-associated gene antigen (vacA), and type two where *cagA* is absent and vacuolating cytotoxin activity is not manifested although *vacA* gene is present. The type one are more strongly pathogenic than the type two and induce a more intense inflammatory response (Xiang et al., 1995).

The presence of *vacA* gene has been reported in all *H. pylori* strains. Some strains now show marked differences in production of vacuolating cytotoxin (Mahaboob et al., 2005). It is assumed that the differences might be due to variations in cytotoxin structure, the region of highest diversity being localized at the N-terminal part of the toxin (corresponding to 2 different toxin signal sequences s1/s2) and the midregion of *vacA* (m1/m2, corresponding to 2 different midregions of the toxin that are required to bind different cell types. The *vacA* genotype s1/m1 unlike s2/m1 (a form of *vacA* associating the signal sequence with a midregion), can vacuolate cells. It has been reported that because of the presence of an additional sequence of 12 amino acids at the N-terminal end of s2 *vacA*, toxin is inactivated (Atherton et al., 1995; Letley et al., 2003). The amino acid sequences
of type m1 and m2 VacA proteins are approximately 65% identical within a region of 250 amino acids (Atherton et al., 1995). Recently, a new vacA polymorphic site, the intermediate (i) region, has been reported where vacuolation assays showed that i-type determined vacuolating activity among s1/m2 strains (Rhead et al., 2007). Another important gene cagA is a highly producers antigenic protein that is associated with a prominent inflammatory response by eliciting interleukin-8 production (Yamaoka et al., 1999a; Gatti et al., 2006; Lim et al., 2009). It is not present in all H. pylori strains (Covacci et al. 1993). cagA is located at one end of the cag-Pathogenicity Island (cagPAI), 40-kb DNA fragment that also encodes molecules constituting the bacterial type IV injection system (Yokoyama et al., 2005). Several studies have followed cagA genotyping for distinguishing various strains of H. pylori (Kaklikkaya et al., 2006; Matteo et al., 2007).

The other important virulence determinant is the iceA gene (induced by contact with epithelium), which has a significant homology to a type II restriction endonuclease and is also associated with H. pylori infection (Peek et al., 1998; Perng et al., 2003; Caner et al., 2007). Two main allelic variants of the gene have been identified; iceA1 and iceA2. Both iceA alleles are flanked by the conserved genes, cysE and hpyLM, but they differ greatly in their genetic organisation and sequence (Figueiredo et al., 2000).

Because the H. pylori is fastidious and microaerophilic, researchers face problems with in vitro culture and growth. In addition, methods developed so far for detecting H. pylori infection, including the H. pylori stool antigen test (Cardinali et al. 2003).
In this chapter we describe a procedure for the simultaneous genotyping of the *H. pylori* virulence determinants, *vacA*, *cagA*, and *iceA*, in paraffin wax embedded specimens. The Nested PCR assay opens up the possibility of testing for genomic *H. pylori* subtypes without the need for microbiological cultures using gastric tissue routinely obtained for diagnostic histopathology. Retrospective studies on wide panels of gastric tissue samples, taken from the files of the archives of pathology, can also be carried out using Nested PCR.

Nested PCR is a modification of polymerase chain reaction intended to reduce the contamination in products due to the amplification of unexpected primer binding sites used to detect infectious pathogens (Saunders and Clewley, 1998). Nested PCR means that two pairs of PCR primers were used for a single locus. The first pair amplified the locus as seen in any PCR experiment. The second pair of primers (nested primers) bind within the first PCR product, and produce a second PCR product that will be shorter than the first one (Figure 3.1). The rationale behind this strategy is that if the wrong locus were amplified by mistake, the probability is very low that it would also be amplified a second time by a second pair of primers. A Nested PCR will produce a detectable amount of PCR product from a very small amount of starting DNA (present in few copies).
The aims:

1) To detect and compare the genotype profiles of \textit{H. pylori} from archived gastric tissue in patients with chronic gastritis (CG) and Intestinal Metaplasia (IM).

2) To evaluate a Nested PCR assay for diagnosing and specific identification of virulent \textit{H. pylori} in archived gastric biopsies and their main virulence genes \textit{cagA}, \textit{vacA}, and \textit{iceA}. 
FIGURE 3.1 Showing summary of the Nested PCR Technique, which uses two sets of primers for amplification of the target gene.

(www.wisconsinlab.com)
Table 3.1 Primers used in Nested PCR for amplification of cagA, vacAs /m, and iceA subtype.

<table>
<thead>
<tr>
<th>Gene Region</th>
<th>Primer</th>
<th>Primer sequence (5'-3')</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>vacA s1/s2</td>
<td>VA1-F</td>
<td>ATG GAA ATA CAA CAA ACA CAC</td>
<td>259 / 286</td>
</tr>
<tr>
<td></td>
<td>VA1-R</td>
<td>CTG CTT GAA TGC GCC AAA C</td>
<td></td>
</tr>
<tr>
<td>m1/m2</td>
<td>VAG-F</td>
<td>CAA TCT GTC CAA TCA AGC GAG</td>
<td>570 / 645</td>
</tr>
<tr>
<td></td>
<td>VAG-R</td>
<td>GCG TCT AAA TAA TTC CAA GG</td>
<td></td>
</tr>
<tr>
<td>cagA</td>
<td>cagA1F</td>
<td>CGT TGA TAA GAA (CT) GA TAG GGA TAA C</td>
<td></td>
</tr>
<tr>
<td></td>
<td>cagA1R</td>
<td>GAT CCC CAA ATT TCT GAA AGC TCT T</td>
<td></td>
</tr>
<tr>
<td></td>
<td>cagA2F</td>
<td>(CT) GA TAG GGA TAA CAG GCA AGC TT</td>
<td>181</td>
</tr>
<tr>
<td></td>
<td>cagA2R</td>
<td>CTG AAA GCT CTT TGT GGA AGA TTC</td>
<td></td>
</tr>
<tr>
<td>iceA1</td>
<td>ice1.1F</td>
<td>ATC ATA AAG ACG GCC GCA AAG AT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ice1.1R</td>
<td>AT (AG) GGG TCA TAT TGA TAA CA (AG) CC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ice1.2F</td>
<td>CCG CAA GGA TGA TT (CT) AAG AGT TTC</td>
<td>218</td>
</tr>
<tr>
<td></td>
<td>ice1.2R</td>
<td>GTC ATA TTG ATA ACA (AG) CC CAC ACA</td>
<td></td>
</tr>
<tr>
<td>iceA2</td>
<td>ice2.1F</td>
<td>CGC TGT TTT TCT AGC GGT GTT TTA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ice2.1R</td>
<td>CAT TGA TCT (AG) TG TTT GTA TGC TCC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ice2.2F</td>
<td>CGG TCT TTT AAT GAG (CT) (AG) G TGG CG</td>
<td>247</td>
</tr>
<tr>
<td></td>
<td>ice2.2R</td>
<td>ATG CTT CTT TGA AAA TGG TAT GGC</td>
<td></td>
</tr>
</tbody>
</table>

bp = base pair
3.2. Results

Eighty-six patients (40 male, 46 female, age range 35-75 years; mean age 52 years) were included in the study. The patients were divided into two groups according to pathogen status, namely an Intestinal Metaplasia (IM) group and a Chronic Gastritis (CG) group. Intestinal Metaplasia is defined as the replacement of the gastric mucosa which, morphologically and histochemically, resembles that of the large, or small intestine.

DNA extraction and Nested PCR were performed on archived cut sections from consecutive paraffin embedded histology blocks of gastric biopsies. To purify DNA, Gene clean III kit was used (Q biogene - UK).

The *H. pylori* DNA integrity and specificity was confirmed by the *ureC* PCR, which rendered the expected 294 bp band from all isolates.

Amplification of the *cagA* gene was observed in 37 of the isolates (43%), and tended to occur more frequently in IM patients than CG patients (68% vs. 17% respectively).

PCR product sizes of *vacA* s and m alleles were used to differentiate them in agarose gels (Figure 3.2 and 3, 3).

The *vacA* subtype combination s2m2 genotypes were the most common allelic combinations of the *vacA* gene and occurred more frequently than the two combinations s1m2, s1m1 (Table 3.2).
The \textit{iceA1} gene was more prevalent than \textit{iceA2}, 38\% and 20\% respectively. However, it was found that 5\% of the isolates were positive for both \textit{iceA1} and \textit{iceA2} and 20\% were negative for both.

The association of the main virulence genes in each strain, was statistically significant and correlation was observed between \textit{vacA s1m1} genotype and \textit{cagA} status ($p=0.001$).

The \textit{cagA} gene was frequently detected, and a significant difference was noted between the two groups: 68\% of specimens with (IM) carried the gene, and 17\% of those with (CG) (Table 3.2).

\textit{vacAs1m1} genotype was detected at a higher frequency in isolates from patients with IM; but the presence of this genotype did not correlate with the CG patients.

The Chronic Gastritis group exhibited the highest frequency of s2m2 strains. Meanwhile, the \textit{iceA1} genotype was significantly associated with IM ($P=0.004$), but not with CG patients.

The prevalence of \textit{cagA}, \textit{vacAs1m1} and \textit{iceA1} was significantly associated with IM patients ($p=0.001$, $p=0.002$, $p=0.001$, respectively).
Figure 3.2. 1.5% Agarose gel electrophoresis after PCR amplification of vacAm1 detection (570 bp) and vacAm2 (645 bp). Lane 1, Molecular size marker ladder, 100bp. Lane 2, positive control vacAm1, and lane 3, positive control vacAm2. Lanes 4, 11, 12, and 13 PCR products (570bp) of positive vacAm1 H.pylori isolates. Lanes 5,6,7,8,9,10 PCR products (645bp) of positive vacAm2 H.pylori isolates.
Figure 3.3. 1.5% Agarose gel electrophoresis after PCR amplification of vacAs1 detection (259 bp) and vacAs2 (286 bp). Lane 1, Molecular size marker ladder, 100bp. Lane 3, positive control vacAs1, and lane 4, positive control vacAs2. Lanes 2, 5, 6, 7, 8, 9, 10, 11, 12 and 13 PCR products (259bp) of positive vacAs1 H. pylori isolates.
Table 3.2 Distribution of *cagA*, *vacA* and *iceA* according to clinical disease.

<table>
<thead>
<tr>
<th>Genes</th>
<th>IM (n=44)</th>
<th>CG (n=42)</th>
<th>Total (n=86)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>cagA</em></td>
<td>30 (68%)*</td>
<td>7 (17%)</td>
<td>37 (43%)</td>
</tr>
<tr>
<td><em>vacA</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>s1m1</td>
<td>16 (36%)*</td>
<td>3 (7%)</td>
<td>19 (22%)</td>
</tr>
<tr>
<td>s1m2</td>
<td>26 (59%)*</td>
<td>3 (7%)</td>
<td>29 (34%)</td>
</tr>
<tr>
<td>s2m2</td>
<td>2 (5%)</td>
<td>36 (86%)</td>
<td>38 (44%)</td>
</tr>
<tr>
<td><em>iceA</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>iceA1</td>
<td>27 (61%)*</td>
<td>6 (14%)</td>
<td>33 (38%)</td>
</tr>
<tr>
<td>iceA2</td>
<td>13 (30%)</td>
<td>4 (10%)</td>
<td>17 (20%)</td>
</tr>
</tbody>
</table>

*P*=<0.05 (IM vs CG)
Figure 3.4. *cagA* status in Intestinal Metaplasia (IM) and Chronic Gastritis (CG) patients
3.3. Discussion

*H. pylori* is the only bacterium that is known to cause malignant diseases such as gastric carcinoma; mucosa-associated lymphoid tissue (MALT) lymphoma, as well as causing gastritis and peptic ulcer disease (Dunn et al., 1997). However, there is a clear discrepancy between the number of infected individuals and patients with clinical symptoms. Although host and environmental factors are considered important (Malaty et al., 1996; Peleteiro et al., 2007; Kim et al., 2008), there is also evidence for a role of specific *H. pylori* genotypes.

The clinical relevance of putative virulence-associated genes of *H. pylori* is still a matter of controversy. This study was designed to detect the *H. pylori* in archived gastric tissue and to compare the genotype profiles of *H. pylori* strains isolated from patients with chronic gastritis and Intestinal Metaplasia of an Irish population.

Infection with certain *H. pylori* genotypes (e.g., cagA+ and vacuolating toxin-producing), is related to the dissociation of the gastric epithelial cells, whereas other variants appear less pathogenic (Atherton 1997; Peek et al., 1997). The *vacA* genotype may also play a role in the efficacy of anti-*Helicobacter* therapy (Cellini et al., 2006; Chaudhuri et al., 2003; Rudi et al., 2002; Sugimoto and Yamaoka, 2009; van Doorn et al., 2000).

Our study described combined genotyping analysis of the main virulence genes of *H. pylori* in a group of Irish patients. It demonstrated the relevance of *H. pylori* cagA, vacA, and *iceA* genes for clinicopathological status and confirmed several earlier findings in other patients from different parts of the world such as the
significant association between the presence of cagA and gastric precancerous lesions (van Doorn et al., 1998; Nogueira et al. 2001; Zambon et al., 2003; Erzin et al., 2006; Plummer et al., 2007; Ladeira et al., 2008). Similarly, our results showed that *H. pylori* strains containing vacA type s1 were found significantly more often in Intestinal Metaplasia patients than type s2 strains, and there was a strong association between the presence of cagA and vacAs1. Also our study confirm the clinically relevant polymorphism in the iceA gene. iceA1 *H. pylori* strains are much more prevalent among patients with IM and iceA1 appears to be marker for gastric Intestinal Metaplasia in our patients.

The combination of the distinct cagA, vacA, and iceA genotypes illustrates the mosaic composition of the *H. pylori* genome. At one side, *H. pylori* strains that are typed as vacAs1m1/cagA+/iceA1 can be considered the most pathogenic and are found in patients with IM. In contrast, strains typed as vacAs2m2/cagA-/iceA2 appear the least pathogenic and occur in chronic gastritis patients.

Genotyping of iceA and cagA offers the most effective combination for identification of patients with IM. However, because cagA, vacAs-region genotypes are so strongly associated, the combination of iceA and vacAs type is almost as effective as the combination of iceA and cagA.

Evidence for the presence of multiple strains based on vacA and iceA genotyping, was found in a considerable numbers of cases. In our study the prevalence of infection with multiple vacA genotypes was much lower (1.1%) compared with results reported from Netherlands (11%), Brazil (13%), Korea (18%), and is in accordance with previous studies on Irish *H. pylori* isolates (Ryan et al., 2001).
With regard to iceA, its prevalence has not been investigated in Ireland previously. It was reported that the iceA allele is related to the development of peptic ulcer disease in the USA (Peek et al., 1998), and in Netherlands (van Doorn et al., 1998) and was only associated with epithelial damage in Portuguese patients (Nogueira et al., 2001), a result that has not been confirmed in other countries, such as Japan and Korea (Yamaoka et al., 1999).

One study showed higher acute inflammatory scores in the gastric mucosa of patients colonized with IceA1-positive strains (Peek et al., 2000), a result that could explain the association between this genotype and gastric diseases as previously reported by other studies (Peek et al., 1998; van Doorn et al., 1998).

In our study, iceA was looked for in samples obtained from all the patients studied (Table 3.2). The iceA1 and iceA2 genotypes were detected in 38% and 20% of our isolates, respectively. This result is similar to the results reported for H. pylori strains from the Netherlands (van Doorn et al., 1998), and Japan (Yamaoka et al., 1999), but the contrasts with results reported from the USA and Colombia (Yamaoka et al., 1999), where iceA2-positive strains are predominant. In our study, the prevalence of iceA1 allele was significantly higher among cases with IM than in cases with CG. However the infection with multiple iceA genotypes was 5% in our H. pylori strains.

The nested PCR analysis of DNA H. pylori isolated from archives of gastric tissue does not require tedious and time consuming culturing of the strains from the biopsies and provides direct evidence for the presence of specific genotypes in the
gastric mucosa. The selected primers (Table 3.1) are based on the sequences of the vacA, cagA, and iceA genes published in the Gene bank database of the NCBI.

Genotyping is very important for prognostication. In this study, the aim was to evaluate the feasibility of a simple method for \textit{H. pylori} genotyping; we performed \textit{H. pylori} detection and genotyping in archived gastric tissue and intended to find out a simple and reliable method for genotyping in the paraffin embedded formalin-fixed gastric tissue.

The procedure was simple and effective for large scale studies, and neither formalin fixation nor paraffin embedding has been noted to interfere with the method of \textit{H. pylori} DNA isolation and genotyping in the biopsies which have been stored for more than five years. This is particularly positive for research purposes, as it can be used on old samples from those patients who might not be available for doing serum genotyping, and also for genotyping in the \textit{H. pylori} negative patients.

\textit{H. pylori} genotyping on archived gastric tissue revealed that \textit{H. pylori} strains with certain combinations of virulence subtypes are associated with IM. Thus, the virulence subtype composition vacA$^S$/cagA$/iceA^1$ occurred mainly in IM, whereas the vacA$^{S2m2}$/cag$^-$/iceA$^2$ were associated in CG patients, showing that the subtype composition has differentiating value. Therefore, when evaluating the possible sequelae of gastric mucosal damage caused by \textit{H. pylori} infection, the composition of virulence subtypes should be taken into account. The simultaneous detection of several virulence genes by the Nested PCR is a convenient technique, with low cost, which is also applicable to paraffin wax embedded tissue.
In conclusion, despite the limitations of the study, analysis of the vacA, cagA, and iceA virulence genes directly from archived gastric biopsy specimens permitted clinically relevant discrimination between _H. pylori_ strains. Each of these individual genes, as well as certain combinations, is significantly associated with the presence of and developing Intestinal Metaplasia.

It is not possible or desirable to treat all _H. pylori_ infected patients worldwide. In the future, combined analysis of vacA, cagA, and iceA genotypes may permit identification of high risk patients who are infected by more pathogenic _H. pylori_ strains. Eventually, patients infected with such strains could be selected for prophylactic anti- _Helicobacter_ treatment to prevent gastro duodenal diseases later in life.
CHAPTER 4

HETEROGENEITY OF cagA, vacA AND babA GENOTYPES AND CLINICAL OUTCOME OF HELICOBACTER PYLORI INFECTION
4.1. Introduction

*H. pylori* is well adapted to the hostile gastric environment, and the majority of infected individuals develop chronic gastritis with further progression to atrophy and intestinal metaplasia, changes which are major risk factors for the development of gastric carcinoma (Correa 1992; Normark *et al.*, 2003). However, it remains unclear why only a minority of infected individuals develops the severe consequences of *H pylori* infection. These variations may be the result of differences in various factors including, host genetics, environmental factors, and the virulence of bacterial strains. A variation in the bacterial virulence has been well documented among different strains of *H. pylori* (Atherton *et al.*, 1995; Clyne *et al.*, 2007). The main virulence factors of *H. pylori* (*cagA, vacA, and babA2*), and their worldwide distributions are displayed in Table 4.1 and Table 4.3, respectively.

One of the most studied virulence factors and a striking difference in the gene contents among *H. pylori* strains is the presence or absence of the *cag* pathogenicity island (*cagPAI*) (Censini *et al.*, 1996; Figueiredo *et al.*, 2002; Dossumbekova *et al.*, 2006). The *H. pylori* *cagPAI* encodes an effector protein, CagA, and a type IV secretion apparatus that translocates CagA into gastric epithelial cells (Backert and Selbach, 2008). *H. pylori* strains harbouring the *cagPAI* are associated with an increased risk of non-cardia gastric cancer or peptic ulcer disease compared to strains that lack the *cagPAI* (Blaser *et al.*, 1995). The correlation between these diseases and presence of the *cagPAI* provides an example of how the clinical outcomes of *H. pylori* infection are determined in part by the genetic characteristics of the strains with which a person is infected. The intact *cagPAI* of *H. pylori* plays a significant role in the pathogenesis of gastritis in humans (Naumann and Crabtree, 2004). Genes within the *cagPAI* are involved in
CagA translocation and associated with increased interleukin (IL)-8 expressions in gastric mucosa (Table 4.2). The cagPAI may be divided into two regions cag I and cag II, by a novel insertion sequence (IS605). There are 16 and 15 open reading frames in cag I and cag II respectively (Censini et al., 1996; Akopyants et al., 1998).

The other potential virulence factor is the vacA gene that encodes for VacA, a secreted vacuolating cytotoxin, which induces a vacuolating cytotoxin effect in a gastric cell line (Leunk et al., 1988). The vacA is a polymorphic gene and both active and inactive forms of the toxin exist (Telford et al., 1994). Strains of H. pylori that express active forms of the toxin are associated with more severe cases of disease (Atherton et al., 1995; van Doorn et al., 1998). A wide range of cellular effects has been attributed to VacA that act on both epithelial and immune cells (Cover and Blanke, 2005). Such activities include the induction of apoptosis (Galmiche et al., 2000), alteration of the process of antigen presentation (Molinari et al., 1998), and inhibition of T cell activation (Gebert et al., 2003; Sundrud et al., 2004). Recently a novel molecular mechanism was identified showing how H. pylori can avoid excessive cellular damage by VacA which can down-regulate CagA effects on epithelial cells (Tegtmeyer et al., 2009). However, the physiological relevance and role in the infectious process of some of the effects of VacA that have been demonstrated in vitro remain to be clarified in vivo (Lu et al., 2005A; Schmees et al., 2006).

H. pylori can bind tightly to the epithelial cells by multiple bacterial surface components (Gerhard et al., 2001). The best characterized H. pylori adhesion molecules that have been described to date are the outer membrane protein BabA, which mediates strong binding of the organism to the fucosylated Leb blood
group antigen, expressed on the surface of gastric epithelial cells and in gastric mucus secretions (Ilver et al., 1998; Linden et al., 2002). Functional BabA is encoded by the babA2 gene. The babA2 gene is identical to the babA1 gene with the exception of an insert of 10 bp with a repeat motif in the signal peptide sequence, which results in the creation of a translational initiation codon and has Leb binding ability (Yamaoka et al., 2000). BabA expression has been shown to be modulated by the types of receptors available in different populations (Aspholm-Hurtig et al., 2004). Heterogeneity among H. pylori strains in expression of the BabA protein may be a factor that contributes to different clinical outcomes among H. pylori infected individuals.

Gastric carcinogenesis is generally believed to be a multistep progression from gastritis to atrophy, intestinal metaplasia (IM), dysplasia, and cancer (Correa, 1992). In a prospective study from Japan H. pylori infected individuals with IM had a 6.5-fold increase in the risk of gastric cancer (Uemura et al., 2001). Hence, the finding of IM acts as an excellent surrogate marker for the higher risk of gastric cancer development.

In Ireland, seroprevalence of H. pylori in a young Irish university population (59%) was 2-3 times the prevalence reported in the same age group in other Western European populations and this high seroprevalence of H. pylori in this young population may have important clinical and economic implications (Sheehan et al., 2004). Some virulence related gene products such as VacA and CagA in the H. pylori isolated strains have been studied in Irish populations (Ryan et al., 2001; Marshall et al., 1999). However, no study in Ireland has simultaneously investigated the prevalence of other putative virulence genes such as cagE, cagT, vacA and babA2, and their relationship to clinical outcomes.
The Aims:-

1- To differentiate *H. pylori* strains using RAPD-PCR, and document cases of colonisation with single and multiple stains in the studied patients.

2- To determine whether the Pathogenicity Island (*cag PAI*) heterogeneity of *H. pylori* strains is related to the clinical outcome of infection.

3- To evaluate the association between the *cagPAI, vacA*, and *babA* status and clinical disease outcomes in Irish adult patients with gastroduodenal pathologies.
<table>
<thead>
<tr>
<th>Virulence factor</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urease</td>
<td>cytoplasmic and surface-associated; liberates NH$_3$ from urea, thereby raising local pH enabling survival.</td>
</tr>
<tr>
<td>CagA</td>
<td>induces cytoskeletal changes following its translocation into epithelial cells; induces inflammation</td>
</tr>
<tr>
<td>Type IV secretion system</td>
<td>transport of CagA into epithelial cells</td>
</tr>
<tr>
<td>CagE</td>
<td>induces IL-8 release from epithelial cells</td>
</tr>
<tr>
<td>VacA (vacuolating cytotoxin)</td>
<td>induces vacuolation and apoptosis in epithelial cells</td>
</tr>
<tr>
<td>BabA (outer membrane protein)</td>
<td>adhesin, binds to Lewis b blood group antigen on gastric epithelial cells</td>
</tr>
<tr>
<td>Lewis x and y antigens on LPS</td>
<td>adhesins, receptors unknown</td>
</tr>
<tr>
<td>Cecropins</td>
<td>antibacterial peptides that may kill competing organisms</td>
</tr>
<tr>
<td>Name of cag gene according to</td>
<td>Involved in</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>-------------</td>
</tr>
<tr>
<td>HP547 cagA</td>
<td>+ +</td>
</tr>
<tr>
<td>ND cagB</td>
<td>+ +</td>
</tr>
<tr>
<td>HP546 cagC</td>
<td>+ +</td>
</tr>
<tr>
<td>HP545 cagD</td>
<td>+</td>
</tr>
<tr>
<td>HP544 cagE (virB4)</td>
<td>+ +</td>
</tr>
<tr>
<td>HP543 cagF</td>
<td>-</td>
</tr>
<tr>
<td>HP542 cagG</td>
<td>+ +</td>
</tr>
<tr>
<td>HP541 cagH</td>
<td>+ +</td>
</tr>
<tr>
<td>HP540 cagI</td>
<td>+ +</td>
</tr>
<tr>
<td>HP539 cagL</td>
<td>+ +</td>
</tr>
<tr>
<td>HP538 cagM</td>
<td>+ +</td>
</tr>
<tr>
<td>ND cagO</td>
<td>ND</td>
</tr>
<tr>
<td>HP536 cagP</td>
<td>-</td>
</tr>
<tr>
<td>HP535 cagQ</td>
<td>-</td>
</tr>
<tr>
<td>ND cagR</td>
<td>ND</td>
</tr>
<tr>
<td>HP534 cagS</td>
<td>-</td>
</tr>
<tr>
<td>HP532 cagT (virB7)</td>
<td>+ +</td>
</tr>
<tr>
<td>HP531 cagU</td>
<td>+ +</td>
</tr>
<tr>
<td>HP530 cagV</td>
<td>+ +</td>
</tr>
<tr>
<td>HP529 cagW (virB8)</td>
<td>+ +</td>
</tr>
<tr>
<td>HP528 cagX (virB9)</td>
<td>+ +</td>
</tr>
<tr>
<td>HP527 cagY (virB10)</td>
<td>+ +</td>
</tr>
<tr>
<td>HP526 cagZ</td>
<td>+</td>
</tr>
<tr>
<td>HP525 cagα (virB11)</td>
<td>+ +</td>
</tr>
<tr>
<td>HP524 cagβ (virD4)</td>
<td>+ +</td>
</tr>
<tr>
<td>HP523 cagγ</td>
<td>+ +</td>
</tr>
<tr>
<td>HP522 cagδ</td>
<td>+</td>
</tr>
<tr>
<td>HP521 cagε</td>
<td>-</td>
</tr>
<tr>
<td>HP520 cagζ</td>
<td>-</td>
</tr>
</tbody>
</table>

+=Positive,  +++= strong positive,  -=Negative,  ND=Not detected
Figure 4.1. Diagram showing the percent distribution of different genetic loci in 140 clinical \textit{H. pylori} isolates.
Table 4.3. Worldwide distribution of the main *H. pylori* virulence factors.

<table>
<thead>
<tr>
<th>Area</th>
<th>vacA alleles prevalence(%)</th>
<th>cagA prevalence(%)</th>
<th>babA2 prevalence(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Europe</td>
<td>48-89 11-51 37 63</td>
<td>66-73</td>
<td>34-72</td>
</tr>
<tr>
<td>America</td>
<td>57-68 16-48 37-44 29-63</td>
<td>57-75</td>
<td>46-69</td>
</tr>
<tr>
<td>East Asia</td>
<td>100 0 41-94 5-55</td>
<td>90-100</td>
<td>80-100</td>
</tr>
<tr>
<td>Africa</td>
<td>80-85 15-20 65-68 30-32</td>
<td>80-95</td>
<td>-</td>
</tr>
<tr>
<td>Gene Amplified</td>
<td>Primer (5’- 3’)</td>
<td>Product size (bp)</td>
<td></td>
</tr>
<tr>
<td>----------------</td>
<td>----------------</td>
<td>------------------</td>
<td></td>
</tr>
<tr>
<td><em>ureC_for</em></td>
<td>AAA GCT TTT AGG GGT GTT AGG GGT TT</td>
<td>294</td>
<td></td>
</tr>
<tr>
<td>_rev</td>
<td>AAG CTT ACT TTC TAA CAC TAA CGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>cagA_for</em></td>
<td>ATA ATG CTA AAT TAG ACA ACT TGA GCG A</td>
<td>298</td>
<td></td>
</tr>
<tr>
<td>_rev</td>
<td>TTA GAA TAA TCA ACA AAC ATC ACG CCA T</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>cagE_for</em></td>
<td>GCG AGC CTA TAA TGA GAA</td>
<td>676</td>
<td></td>
</tr>
<tr>
<td>_rev</td>
<td>AAC TGC CTA GCG TAA TAT CAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>cagT_for</em></td>
<td>ATG AAA GTG AGA GCA AGT GT</td>
<td>843</td>
<td></td>
</tr>
<tr>
<td>_rev</td>
<td>TCA CTT ACC ACT GAG CAA AC</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>(babA2)</em>_for</td>
<td>AAT CCA AAA AGG AGA AAA AGT ATG AAA</td>
<td>607</td>
<td></td>
</tr>
<tr>
<td>_rev</td>
<td>CTT TGA GCG CGG GTA AGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>babA2_for</em></td>
<td>AAT CCA AAA AGG AGA AAA AGT ATG AAA</td>
<td>830</td>
<td></td>
</tr>
<tr>
<td>_rev</td>
<td>TGT TAG TGA TTT CGG TGT AGG ACA</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Emty site_for</em></td>
<td>ACA TTT TGG CTA AAT AAA CGC TG</td>
<td>562</td>
<td></td>
</tr>
<tr>
<td>_rev</td>
<td>GGT TGC ACG CAT TTT CCC TTA ATC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Zambon primers (Zambon et al., 2003).
Table 4.5. RAPD-PCR fingerprinting primers used in this study

<table>
<thead>
<tr>
<th>Primer  (^{(1)})</th>
<th>Primer sequence (5' - 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>1254</td>
<td>CCGCAGCCAA</td>
</tr>
<tr>
<td>1283</td>
<td>GCGATCCCCA</td>
</tr>
</tbody>
</table>

1) Primer nomenclature according to Akopyanz et al., 1992a and Carroll et al., 2003.
4.2. Results

**RAPD-PCR analysis of H. pylori isolates.**

RAPD-PCR fingerprinting was carried out on the paired isolates (Antrum/Corpus) to determine whether each pair represented isolates with identical (ancestrally related) or different (mixed infection) profiles.

We performed RAPD-PCR fingerprinting on 12 *H. pylori* isolates from antral and corpus biopsies from six patients, three with (IM), and three with (CG), with the use of two sets of primers (Table 4.5), considered to be highly discriminating.

RAPD-PCR revealed that the six pairs which displayed identical fingerprint profiles and showed similar RAPD patterns with each of the primers used (Figure 4.2).

The profiles of each pair differed from those of other pairs, however, within each pair interpair similarity was noted in number of amplimers (bands) generated by the two primers (primer 1283, and 1254).

The RAPD fingerprinting with the two primers yielded 4-8 bands on the agarose gels (Figure 4.3). The diversity of the *H. pylori* strains was exhibited by a unique band pattern in each of the clinical isolates.

The RAPD-PCR testing was repeated three times on different days to avoid any genetic changes that may have occurred due to in vitro handling of the strains. These results showed stable and unique patterns at three different time points.
Detection of *H. pylori* genotypes.

*H. pylori* was successfully cultured from one-hundred and forty Irish adult dyspeptic patients. Distribution of the three groups of IM, CG, and PU patients, according to the sex and age are presented in Table 4.6.

The DNA integrity and specificity was confirmed by the ureC PCR, which rendered the expected 294 bp band from all isolates.

Presence of the Pathogenicity Island in *H. pylori* strains was determined by using primers for cag I (cagA, cagE) and cag II (cagT). Specific primers for the cag empty site were used to confirm the absence of the cagPAI. Samples positive for cagA, cagE, and cagT and cag empty site primers were regarded as harbouring mixed infections with cagPAI positive and cagPAI-negative strains and were excluded from further analysis.

The mixed infection by cagPAI-positive and cagPAI-negative strains was found in only 8.5% (6% in IM, 12% in CG, and 7.5% in PU) patients.

Details of the percent distribution of different genetic loci have been depicted in (Figure 4.1).

**cag I markers:** -

In 140 *H. pylori* -positive patients studied, amplification of the cagA gene was visualized as a band of 298 bp (Figure 4.4), and present in 91 positive strains (65.5%). The cagA gene was detected in 44 of 50 (88%) strains isolated from patients with IM, 17 of 50 (34%) with CG, and 30 of 40 (75%) with PU. Statistically
these differences were highly significant for the presence of cagA gene and clinical diseases (Table 4.7).

The most frequently detected genes by PCR in Irish strains were cagE gene, found in 87% (122/140) of clinical isolates. The prevalence of the cagE gene was 90% (45/50) in strains from patients with IM, and 82% (41/50) with CG and 90% (36/40) with PU. However no association was detected between the presence of cagE gene and clinical outcomes.

cag I I markers:
The cagT gene as a cagII marker was present in 67% (94/140) of clinical isolates. The cagT gene was detected in 90% (45/50) of strains from patients with IM, and in 345 (17/50) with CG, 80% (32/400) with PU. As for the cagA gene, there was a statistically significant relationship between the presence of cagT gene and the diseases status.

Combination of cagi and cagII with clinical outcome:
The coexistence of cagi and cagII (i.e., positive for cagA*, cagE*, and cagJ*: triple-positive) was found in 54% (75/140) of the isolates (Table 4.7). These triple-positive strains were detected in 84% (42/50) of isolates from patients with IM, 14% (7/50) from CG patients and 65% (26/40) of patients with PU.

H. pylori strains lacking both cagi and cagII (i.e., negative for cagA-, cagE-, and cagT-: triple-negative) were detected in 12% (17/140) of the isolates including 10% (5/50) of isolates from IM,18% (9/50) of CG patients and 8% (3/40) form PU patients.
At least 29% (40/140) of *H. pylori* strains possessed the partially deleted cagPAI (*cagA*-*cagE* or *cagA*cagT*), and there was a statistically significant relationship between the combinations of cagi and cagII and clinical outcomes (p<0.05).

**Detection of vacA alleles**

PCR product sizes of vacA s and m alleles were used to differentiate them in agarose gels (Figure 3.3 and 3.4).

The most virulent vacAs1 allele was predominantly present in 89% (125/140) of clinical isolates (Table 4.8), and was visualized as a band of 259 bp on agarose gel electrophoresis. 86% of these were s1a and 3% were s1b, no amplification of s1c was noted in the isolates, whereas 10% of isolates had the vacAs2 genotype.

The middle region of the vacA gene was detected in 99.5% of the isolates, m1 and m2 genotypes were distributed in 35% and 64%, respectively. Hybrids corresponding to m1m2 alleles were not detected, but non-typable alleles were detected in 1% of the strains isolated.

The vacA subtype combination s1m1 and s2m2 genotypes were the most common allelic combinations of the vacA gene among the isolates and only 1% of *H. pylori* strain harbour the s2m1 genotypes. A summary of these results according to the clinical outcome and the vacA subtype s and m alleles is shown in Table 4.8.
Combination of *cagA* and *vacA* genotypes

The association between *cagA* presence and *vacA* alleles is described in Table 4.9. Of the 91 *H. pylori* that were positive for *cagA*, 40 strains (29%) were associated with the toxin-producing *vacAs1m1*, 36% (51/140) *cagA*-positive were *vacAs1m2*, and *s2m2* was only related to *cagA*-negative *H. pylori* strains isolates 9% (12/140). The CG group exhibited the highest frequency of *cagA*-negative/*vacAs2m2* strains.

*babA2* status and clinical outcome

The *babA2* gene was detected in only 47% (66 strains) of our *H.pylori* collection. Of 140 *H.pylori* positive, 42 (84%) patients with IM were positive for *babA2*, compared with 14 (28%), and 10 (25%) strains from CG and PU patients, respectively. A significant association (p < 0.05) was found between *babA2* presence and IM. The gene association is described in Table 4.10.

Combination of *cagA*, *vacA* and *babA2* genotypes

On examining the association of main virulence genes in each strain, a statistically significant correlation was observed between *vacAs1m1* allele, *cagA* status and *babA2* genotype (P= 0.035), and most *s2m2* strains carried a *cagA*-negative genotype (Table 4.9). Additionally, 40 (29%) isolates were classified as type 1 (*cagA*/*vacAs1m1*) *H. pylori* strains and 61 (44%) were triple positive (*cagA*/+/*vacAs1m1*/+/babA+) *H. pylori* strains (Table 4.10).
Table 4.6. Distribution of 140 patients according to sex and age.

<table>
<thead>
<tr>
<th>Disease</th>
<th>10-20</th>
<th>20-30</th>
<th>30-40</th>
<th>40-50</th>
<th>50-60</th>
<th>Upper 60</th>
<th>M</th>
<th>F</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>IM</td>
<td>0(^1)</td>
<td>2</td>
<td>2</td>
<td>9</td>
<td>16</td>
<td>21</td>
<td>28</td>
<td>22</td>
<td>50</td>
</tr>
<tr>
<td>CG</td>
<td>3</td>
<td>10</td>
<td>10</td>
<td>13</td>
<td>5</td>
<td>9</td>
<td>26</td>
<td>24</td>
<td>50</td>
</tr>
<tr>
<td>PU</td>
<td>2</td>
<td>4</td>
<td>5</td>
<td>11</td>
<td>4</td>
<td>14</td>
<td>26</td>
<td>14</td>
<td>40</td>
</tr>
</tbody>
</table>

IM = Intestinal Metaplasia, CG = Chronic Gastritis, PU = Peptic Ulcer
1) Number of patients
Table 4.7. Correlation between the clinical disease and the structure of 
cagPAI of (140) H.pylori infected subjects

<table>
<thead>
<tr>
<th></th>
<th>(IM) no=50 (%)</th>
<th>(CG) no=50 (%)</th>
<th>(PU) no=40 (%)</th>
<th>(Total) no=140 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>cagA</em></td>
<td>44(88)*</td>
<td>17(34)</td>
<td>30(75)</td>
<td>91(65)</td>
</tr>
<tr>
<td><em>cagE</em></td>
<td>45(90)</td>
<td>41(82)</td>
<td>36(90)</td>
<td>122(87)</td>
</tr>
<tr>
<td><em>cagT</em></td>
<td>45(90)*</td>
<td>17(34)</td>
<td>32(80)</td>
<td>94(67)</td>
</tr>
<tr>
<td>ES</td>
<td>3(6)</td>
<td>6(12)</td>
<td>3(8)</td>
<td>12(9)</td>
</tr>
<tr>
<td><em>cagA</em> <em>cagE</em></td>
<td>44(88) *</td>
<td>17(34)</td>
<td>29(73)</td>
<td>90(64)</td>
</tr>
<tr>
<td><em>cagA</em> <em>cagT</em></td>
<td>45(90)*</td>
<td>13(26)</td>
<td>29(73)</td>
<td>87(62)</td>
</tr>
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<td><em>cagA - cagE</em></td>
<td>1(2)</td>
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<td>3(8)</td>
<td>7(5)</td>
</tr>
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<td><em>cagA</em> - <em>cagT</em></td>
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<td>30(60) *</td>
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</tr>
<tr>
<td><em>cagA</em> <em>cagE</em> <em>cagT</em></td>
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<td>7(14)</td>
<td>26(65)</td>
<td>75(54)</td>
</tr>
<tr>
<td><em>cagA</em> <em>cagE</em> <em>cagT</em></td>
<td>5(10)</td>
<td>9(18)</td>
<td>3(8)</td>
<td>17(12)</td>
</tr>
</tbody>
</table>

ES= the Empty Site

*p=<0.05
Table 4.8. Correlation between *vacA* subtype and clinical outcomes.

<table>
<thead>
<tr>
<th>vacA genotype</th>
<th>IM (n=50)</th>
<th>CG (n=50)</th>
<th>PU (n=40)</th>
<th>Total (n=140)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. (%)</td>
<td>No. (%)</td>
<td>No. (%)</td>
<td>No. (%)</td>
</tr>
<tr>
<td>s1</td>
<td>48 (96)</td>
<td>39 (78)</td>
<td>38 (95)</td>
<td>125 (89)</td>
</tr>
<tr>
<td>s1a</td>
<td>44 (88)</td>
<td>39 (78)</td>
<td>38 (95)</td>
<td>121 (86)</td>
</tr>
<tr>
<td>s1b</td>
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<td>0</td>
<td>4 (3)</td>
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<tr>
<td>s1c</td>
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<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>s2</td>
<td>2 (4)</td>
<td>11 (22)</td>
<td>1 (3)</td>
<td>14 (10)</td>
</tr>
<tr>
<td>m1</td>
<td>34 (68)</td>
<td>2 (4)</td>
<td>13 (33)</td>
<td>49 (35)</td>
</tr>
<tr>
<td>m2</td>
<td>16 (32)</td>
<td>48 (96)</td>
<td>26 (65)</td>
<td>90 (64)</td>
</tr>
<tr>
<td>s1m1</td>
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</tr>
<tr>
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<td>vacA genotype</td>
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<td>CG (n=50)</td>
<td>PU (n=40)</td>
<td>Total (n=140)</td>
</tr>
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<tr>
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<td>cagA⁻</td>
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<td>5(10)</td>
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<tr>
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<td>28(56)</td>
<td>4(8)</td>
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<tr>
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<tr>
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<td>- (0)</td>
<td>1(2)</td>
<td>- (0)</td>
<td>- (0)</td>
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</table>

1) Total positive number of the gene.
2) Number in percentage.
<table>
<thead>
<tr>
<th>Genotype</th>
<th>(IM) n=50 (%)</th>
<th>(CG) n=50 (%)</th>
<th>(PU) n=40 (%)</th>
<th>(Total) n=140 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cagA</td>
<td>44(88)*</td>
<td>17(34)</td>
<td>30(75)</td>
<td>91(65)</td>
</tr>
<tr>
<td>cagE</td>
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<td>36(90)</td>
<td>122(87)</td>
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<td>32(80)</td>
<td>94(67)</td>
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<td>vacAs1</td>
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<td>39(78)</td>
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<td>13(33)</td>
<td>49(35)</td>
</tr>
<tr>
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<td>48(96)</td>
<td>26(65)</td>
<td>90(64)</td>
</tr>
<tr>
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<td>2(4)</td>
<td>13(33)</td>
<td>47(34)</td>
</tr>
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<td>37(74)</td>
<td>26(65)</td>
<td>79(56)</td>
</tr>
<tr>
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<td>1(3)</td>
<td>12(9)</td>
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<td>babA2</td>
<td>42(84)*</td>
<td>14(28)</td>
<td>10(25)</td>
<td>66(47)</td>
</tr>
<tr>
<td>cagA*, vacAs1*, babA2*</td>
<td>41(82)*</td>
<td>10(20)</td>
<td>10(25)</td>
<td>61(44)</td>
</tr>
</tbody>
</table>

*p<.05
Figure 4.2 RAPD-PCR profile using primer 1254 from six paired (Antrum and Corpus) and one Antrum of *H. pylori* clinical isolates.
Figure 4.3 RAPD-PCR profiles using primer 1283 from six paired (Antrum and Corpus) and one Antrum of *H. pylori* clinical isolates.
Figure 4.4 Agarose gel electrophoresis of cagA gene by PCR. PCR products (298 bp) from positive cagA *H. pylori* isolates (lanes 1, 2, 3, 4, 5, 6, 8, and 9) and negative *H. pylori* isolates (absence of PCR products, lanes 10, and 12). Lane 11, positive control; lane 13, negative control. Lane 1, Molecular size marker ladder, 50bp.
4.3. Discussion.

*H. pylori* is cosmopolitan and one of the most frequent world-wide infections. The pathogen, a genetically diverse species with a high DNA recombination rate, may be involved in a complex variety of diseases in infected patients (Dunn *et al.*, 1997; Rokkas *et al.*, 2007). Moreover, 12 - 18% of each strain genome accounts for strain specific genes (Akopyants *et al.*, 1998; Salama *et al.* 2000; Lin *et al.*, 2001).

Although a number of studies have investigated *H. pylori* virulence genes in our population (Marshall *et al* 1999; Ryan *et al.* 2001; Carroll *et al.*, 2003), a definite virulence marker for *H. pylori* -related disease has not yet been identified. The identification of a disease-specific *H. pylori* virulence factor with a predictive value for the outcome on infection could be useful in clinical practice, as it would help to identify patients who are at higher risk of developing a serious disease related to *H. pylori*. Our aim in the present study was to determine the prevalence of virulence genes of *H. pylori* in gastric biopsy samples obtained from IM, CG, and PU patients using PCR method of DNA amplification.

In our study *H. pylori* cagPAI, vacA, and babA2 gene presence was studied in a series of 140 *H. pylori* positive patients. Evidence for the presence of multiple strains was based on genotyping within cagPAI (cagA, cagE, cagT, and empty site region) and was found in 9% of the cases. The prevalence of mixed *H. pylori* infection in the clinical setting seems to be variable, ranging from 0% to 85% in different populations (Yakoob *et al.*, 2001; Berg *et al.*, 1997; Enroth *et al.*, 1999; Owen *et al.*, 1993; Jorgensen *et al.*, 1996; Sheu *et al.*, 2008). The presence of mixed infection due to *H. pylori* strains within a single host has important clinical consequences. Therefore, the effect of mixed *H. pylori* infection on gastric
histology is a topic worthy of further investigation. In our study we evaluated whether the mixed infection observed in some of our patients was the result of multistrain \textit{H. pylori} infection and not a mere expression of true genomic modifications within the same strain, such as deletions, mutations, and recombinations. These phenomena are commonly described in these species (Jenks et al., 1998; Han et al., 2000; Kersulyte et al., 1999). We performed RAPD-PCR, because this technique yields strain specific profiles based on presence of particular sequences, which vary with different primers, within the entire bacterial genome. These techniques showed that the antrum and the corpus region of the stomach of each patient carrying mixed colony were infected with a single \textit{H. pylori} strain. The profile of \textit{H. pylori} cagPAI genes shows great variability worldwide. Fifty per cent of strains from West India are devoid of the entire island of genes (Kauser et al., 2004), 32% in France (Audibert et al., 2001), 9% in Sweden (Nilsson et al., 2003) and the USA (Hsu et al., 2002) and 1% in Japan (Ikenoue et al., 2001). In our study population 12% of the \textit{H. pylori} strains showed complete deletion of the cagPAI genes (cagA, cagE, cagT). The Chronic Gastritis patients had a higher frequency of deletions in the cagPAI 18% (Table 4.7). Total deletion of cagPAI has been associated with lower production of IL-8 in vitro (Audibert et al., 2001; Hsu et al., 2002). However, association between the presence of any genes of the cagPAI or the intactness of the cagPAI with clinical outcomes has been contradictory; some studies have shown an association (Jenks et al., 1998; Ikenoue et al., 2001; Kidd et al., 2001; Nilsson et al., 2003; Pacheco et al., 2008), while others have not (Hsu et al., 2002; Chomvarin et al., 2008). In our study most of the strains from IM and PU patients retained cagA (88%, 75%), cagE (90%, 90%) and cagT (90%, 80%) respectively. \textit{H. pylori} isolates with partially or totally deleted cagPAI, which lacked both cagE and cagT, were found more frequently in patients with CG as compared
with IM or PU patients (Table 4.7). Our results are similar to another study in English patients where the majority of ulcer disease strains retained the \textit{cagE} and \textit{cagT} genes (Kauser \textit{et al.}, 2005). \textit{CagT} is found at the base of the outgrowing pilus of the type IV secretion system and is supposed to be responsible for binding to a cellular receptor inducing IL-8 and for ejecting CagA (Rohde \textit{et al.}, 2003). \textit{CagE} is absolutely necessary for transcription factor NF-\textit{xB} activation, which mediates IL-8 secretion (Glocker \textit{et al.}, 1998).

The \textit{cagA} gene was detected in 65.5\% of our patients, this is in a concordance with previous Irish studies where \textit{cagA} was detected in 69\% and 76\% of the patients, respectively (Dundon \textit{et al.}, 2000; Ryan \textit{et al.}, 2001). These findings somewhat differ from another Irish study which found that all of the 74 Irish \textit{H. pylori} isolates contained the \textit{cagA} gene. This difference may be due to the fact that \textit{H. pylori} isolates were from patients experiencing peptic ulcer disease only and did not include asymptomatic patients (Carroll \textit{et al.}, 2003). However our observations were confirmed in other European countries and USA, where the \textit{cagA}-positive \textit{H. pylori} is reported to account for 60 to 70 \% of \textit{H.pylori} strains (Andreson \textit{et al.}, 2002; Basso \textit{et al.}, 2008; Schneller \textit{et al.}, 2006 Rudi \textit{et al.}, 1998). Reports from East Asian countries have shown that more than 90\% of \textit{H. pylori} are \textit{cagA}-positive irrespective of the disease presentation (Ito \textit{et al.}, 1997; Ko \textit{et al.}, 2008). Such differences in the prevalence of \textit{cagA} positivity could not be explained precisely; however, they have been attributed to the genetic heterogeneity and from differences in the geographic locations (Covacci \textit{et al.}, 1999; Yamaoka \textit{et al.}, 2002). Our results revealed that the majority of IM (88\%), and PU (75\%), patients were infected with \textit{cagA}-positive strains as opposed to CG patients (34\%) as shown in Table 4.7. These findings substantiate the role of \textit{cagA} as a marker for
increased virulence and are in agreement with studies from other European countries (Arents et al., 2001; Stephens et al., 1998; Zambon et al., 2003; Chiarini et al., 2009). Further, in subjects with partial cagPAI, 2% showed IM, 50% showed CG, 18% showed PU (Table 4.7) suggesting the role of other virulence genes. Hence, we can suggest that the cagPAI is a strong virulent marker in disease pathogenesis in our population.

Analysis of H. pylori isolates from diverse geographic locations also showed high variability in the vacA gene (van Doorn et al., 1999; Dundon et al., 2001; Wen and Moss, 2009). The most common signal sequence and midregion alleles were s1a and m2 respectively (Table 4.8), which is in accordance with previous studies of Irish H. pylori isolates (Ryan et al., 2001; Carroll et al., 2003), and with other European countries. In our study, the cagA-positive status was strongly associated with vacAs1a in IM and PU and these findings are similar to previous studies from European countries (Strobel et al., 1998; van Doorn et al., 1999; Zambon et al., 2003). In contrast, however, such relationships were not reported in studies from East Asia (Ito et al., 1997; Yamaoka et al., 1999; Zheng et al., 2006; Chomvarin et al., 2008). Overall, it may be concluded that the cagA-positive and vacAs1am2 genotypes are typically observed in European countries, but not in H. pylori strains from East Asia, and related to the clinical outcomes in infected populations, while the cagA-negative and vacAs2 genotypes are considered the least virulent strains.

Also in our study none of the H. pylori strains was positive for cagA or vacAs1c genotypes, whish is typically observed in strains from East Asia and considered the most virulent strains. This might be one of the reasons for the low prevalence of gastric cancer in our population compared to a higher prevalence of gastric cancer
in the East Asian countries. Adherence of *H. pylori* to epithelial cells is an important step in the development of gastroduodenal pathologies. Many *H. pylori* express a blood group antigen-binding adhesin (BabA), an adhesin that mediates attachment of *H. pylori* to gastric epithelium. Some studies found a low prevalence (36 to 38.9%) of *H. pylori* harbouring *babA2* gene (Podzorski et al., 2003; Zheng et al., 2006), whereas other studies in Japanese and Thai patients found a high prevalence (84.9 to 92%) of strains harbouring *babA2* gene (Mizushima et al., 2001; Chomvarin et al., 2008). In our study, we detected *babA2* gene in 47% of *H. pylori* positive isolates (Table 4.10). These results show that the prevalence of *babA2* gene varies among patients and correlation between the presence of *babA* and IM patients (84%) remains controversial. These results are similar to other studies which suggested that expression of *babA2* gene is associated with increased gastric inflammation and intestinal metaplasia (Prinz et al., 2001; Yu et al., 2002; Zambon et al., 2003). However other studies indicate that the *babA2* gene is not necessary for *H. pylori* for induction of increased inflammatory response or higher incidence of IM, however other adhesion molecules other than BabA2, like SabA (Sialic acid binding adhesin), may be responsible (Schneller et al., 2006). In some isolates, the *babA* gene was not expressed or was replaced by *babB*, which encoded a related protein. Absence of *babA* and duplication of *babB* was seen in *H. pylori* isolates derived from human clinical samples, suggesting that the gene conversion is of relevance to the human host and might reflect diverging selective pressures for adhesion either across hosts or within an individual (Colbeck et al., 2006). The changes in *babA* and *babA* expression represent a dynamic response in the outer membrane of *H. pylori*. These changes facilitate its adherence to the gastric epithelium, while promoting chronic infection
by extensive genotypic diversity which is displayed by the human *H. pylori* isolates and within a strain colonizing an individual patient (Dereuse and Bereswill 2007).

In summary, our results show that *H. pylori* isolate including type 1 and triple-positive strains were associated with a higher degree of gastro duodenal lesions. Our data indicate that type 1 and triple-positive strains increase the risk of developing IM in dyspeptic patients.
CHAPTER 5

*Helicobacter pylori* Related Gastroduodenal Pathologies: Association with Plasticity Region and Novel Virulence Factors
5.1. Introduction

*H. pylori* infection leads to ongoing structural and functional damage to gastric mucosa and is a major cause of gastric malignancy. Some bacterial strains express virulence factors that increase the risk of clinical disease outcomes. Putative virulent genes for *H. pylori* include the cagPAI, vacA, iceA, and babA. Although these virulence factors have been associated with increased risks of a clinical outcome, but none has shown disease specificity. It was recently suggested that some genes, located outside the cagPAI such as oipA, hrgA, vacAi -region and plasticity region genes are strain-specific and might be associated with clinical outcomes among *H. pylori* isolates (Ando et al., 2002; de Jonge et al., 2004; Lu et al., 2005; Rhead et al., 2007; Santos et al., 2003; Yamaoka et al., 2002; Yamaoka et al., 2006).

Comparison of the genomic contents of two *H. pylori* strains (26695 and J99) revealed a family of thirty - two genes encoding outer membrane proteins (OMPs). In addition to the cagPAI, the presence of regions with different G+C content may also represent potential pathogenicity island (PAIs). Among these (eight in 26695 and nine in J99) one so-called "Plasticity region", is a large region of 45Kb in strain J99 and 68Kb in strain 26695; it encodes 38 genes in J99, of which 33 are absent in 26695 (Alm et al., 1999; Tomb et al., 1997).

The oipA (outer inflammatory protein) gene which encodes one of the outer membrane proteins is an inflammation-related gene located approximately 100 Kb from the cagPAI on the *H. pylori* chromosome (Yamaoka et al., 2000). It is polymorphic, due to the presence of a CT dinucleotide repeat in the 5' region (Signal sequence coding region), suggesting that a slipped strand mispairing (SSM)
regulatory mechanism governs its expression. *H. pylori* contain either a functional or non-functional *oipA* gene. The functionality depends on the number of CT repeats in the signal sequence coding region. The functional *oipA* gene is contained in 6 and 9 CT dinucleotide repeats and in this configuration, the peptide sequence remains "in frame" such that the gene status could be considered as "on". A non-functional *oipA* gene contained in 5 and 7 CT dinucleotide repeats that keeps the peptide "out-of-frame" in a status considered as "off". The functional *oipA* gene is strictly correlated with *cagPAI*, and its functionality has been recorded in strains infecting subjects with severe gastric inflammation, duodenal ulcer, and gastric cancer (Yamaoka *et al.*, 2002; Zambon *et al.*, 2003).

A potential marker for *H. pylori* restriction endonuclease - replacing gene (*hrgA*) in conjunction with *cagA* that identifies individuals with gastric cancer has been reported (Ando *et al.*, 2002). The *hrgA* seems more prevalent among the Western countries than in Asians and its prevalence being common among gastric cancer patients in Asians compared to those with benign disease (Ando *et al.*, 2002).

Recently a new polymorphic region in *vacA*, termed as intermediate (i) region, has been identified which is located between the s and m regions. The (i) region encodes the part of p33 VacA subunit and their 2 types, i1 (vacuolating) and i2 (nonvacuolating) are identified (Rhead *et al.*, 2007). The study showed that the 'i' region was directly involved in toxicity by exchange mutagenesis experiments. A PCR-based typing system for this region was developed and showed that in an Iranian population the i1 genotype was a better marker for cancer-associated strains as compared with s1 or m1 (Rhead *et al.*, 2007), however in another study it was considered as a risk factor for DU (Basso *et al.*, 2008).
Recently a series of studies showed that certain genes or combination of genes within the plasticity region may play important roles in the pathogenesis of *H. pylori* associated gastro duodenal diseases (Arachchi et al., 2008; de Jonge et al., 2004; Lehours et al., 2004; Lu et al., 2005; Santos et al., 2003). Most of these studies have focused on the plasticity region in strain J99 (*jhp0914-jhp0961*), *jhp947* gene and the duodenal ulcer promoting (*dupA*) gene. The *jhp947* gene is reported to have association with an increased risk of both duodenal ulcer and gastric cancer (de Jonge et al., 2004), whereas the *dupA* gene, which encompasses *jhp0917* and *jhp0918* is associated with an increased risk of duodenal ulcer and protection against gastric cancer (Lu et al., 2005). In addition, recent studies showed that approximately 10-30 % of *H. pylori* isolates possess a 16.3 kb type IV secretion apparatus (tfs3) in the plasticity region which has gained significant attention, although its role is not clear (Kersulyte et al., 2003; Alvi et al., 2007).

**The Aims:**

1. To determine the prevalence of *Helicobacter pylori* restriction endonuclease-replacing (*hrgA*) among *H. pylori* clinical isolates

2. To investigate the relationship between the presence of *dupA* and the clinical outcomes and to determine whether this association is independent of other virulence factors such as *cagA*, *vacA*, and *babA2*.

3. To study the association of both new and established *H. pylori* virulence markers (*vacA* s, m, i region polymorphisms, *cagA* status and clinical outcomes).
5.2 Results

A- Amplification of plasticity zone genes:

The prevalence of the plasticity zone genes (JHP940, JHP947, and HP986 as well as jhp0917, jhp0918 (dupA) in the H. pylori strains isolated from 140 adult patients with gastro duodenal disease were determined by positive PCR amplification. The PCR positive strains were tested at least twice for each sample and showed identical results.

1-Distribution of JHP940, JHP947, and HP986:

The JHP940 gene was found in only 1 of 140 H. pylori strains, this was from a patient with IM. This gene was not found in strains isolated from CG or PU. H. pylori strains also showed low frequency for HP986 gene (29 of 140) and was present in only 4 of 40 (10%), 10 of 50 (20%), and 15 of 50 (30) strains isolated from patients with PU, CG, and IM, respectively. On the other hand, the JHP947 gene was found in 52 of 140 (37%) (Table 5.4).

The association between JHP947 and disease outcome:

Thirty (60%) out of the 50 patients with IM, 16 (32%) out of the 50 patients with CG, and 6 (15%) out of the 40 patients with PU were colonized by a JHP947-positive strain. When patients with IM and CG, PU were compared, the presence of the JHP947 gene was positively associated with IM (P<0.05).

The cagPAI-positive / JHP947 and disease outcome:

Among the 52 H. pylori strains harbouring the JHP947 gene, 38 (73%) were also cagPAI-positive. Both the cagPAI-positive and JHP947 were present in a single strain in 38 of 52 (27%) of the H. pylori strains, and this was
significantly associated with the presence of IM (P=<0.05). Of the 38 positive strains, 27 (71%) were IM patients and 13 and 16% were CG and PU, respectively (Table 5.4).

2- Detection of the jhp0917, jhp0918 (dupA):

Overall both jhp0917 and jhp0918 (dupA) were detected in 60 (43%) of *H. pylori* strains isolated. The absence of both genes was seen in 76 (54%) strains. Our results showed that the presence of the jhp0917 and jhp0918 genes were strongly linked (p<0.001). All the *H. pylori* strains harbouring the jhp0917 and jhp0918 genes are considered to be dupA-positive. Three *H. pylori* strains (2%) possessed jhp0917 positive/jhp0918 negative genotype, and one strains (1%) had jhp0917 negative/jhp0918 positive genotype. These four *H. pylori* strains were classified as dupA negative (Table 5.8). The distribution of dupA in relation to clinical outcome and the frequency of dupA in Ireland and in other reported countries are shown in Figure 5.1, and Table 5.9. respectively.

The prevalence of dupA and other pathogenicity associated genes:

The combinations of dupA-positive and other virulence genes are summarized in Table 5.7. The positivity rate of cagA, babA2, cagPAI, vacAs1a, vacAs1m2, and the triple-positive (cagAs1m1babA2) with dupA-positive were detected in 41 (29%), 30 (21%), 32 (23%), 56 (40%), 39 (28%), and 17 (12%), respectively. The presence of dupA was not associated with any other virulence genes for three groups of our patients with IM, CG, and PU (p>0.50).
**dupA status and *H. pylori*-associated diseases:**

The prevalence of the *dupA* gene was high in strains from CG 25 of 50 (50%), compared with those from PU, 14 of 40 (35%), or IM (21 of 50 (42%), however the difference was statistically not significant (*p*=0.2). No association between *dupA* status and peptic ulcer was observed in *H. pylori* strains. Similarly when patients with IM and PU were compared, no significant difference between *dupA* status was observed (*p*=0.21). The distribution of *dupA* gene in relation to clinical outcome in Ireland and other reported countries is shown in Table 5.9.

**B- The functional status of *oipA* gene:**

The primers used for this new PCR amplified the *oipA* gene in all strains, based on the CT dinucleotide repeats present in the signal sequence coding region of the *oipA* gene (Table 5.2). The *oipA* functional status was performed in only 12 *H. pylori* positive strains isolated from IM patients. Nine *H. pylori* strains (75%) were considered to be *oipA* "on", and three (25%), were considered to be "off". The number of CT dinucleotide repeats in IM strains ranged from 6 to 9 CT repeats. Among the 12 *H. pylori* strains used, the sequence of the signal peptide coding region, numbers of CT repeats, and gene status is shown in Table 5.6.A and Table 5.6.B, respectively.

**oipA functional status and other virulence genes:**

Overall *oipA* "on" status was closely related to *cagA*, *cagPAI*, *vacAs1am2*, and *babA2* genes. However *vacAs1m2* was more prevalent (67%) than *vacAs1m1* (17%), or *vacAs2m1* (8%). Two of the three *H. pylori* with functional status *oipA* "off" were closely associated with *cagA*, *cagPAI*, and *babA2* negative genes, one
was with vacAs1bm2, and the other with vacAs2m1. However one was cagA, cagPAI, and babA2 positive (Table 5.11)

C- Prevalence of vacA1-region:
Using the primers VacF1, C1R for vacA1 region, and VacF1 and C2R for vacA12 region, the PCR method were performed successfully for the amplification of the vacA1-region in all of the 140 H.pylori-positive strains. Genotyping of the 4 cases (one IM, two CG, and one PU) showed mixed strains infection in both 11 and 12 combinations (3%). These cases were not included in the subsequent association analysis. The vacA1 region genotype was detected in all of the H. pylori isolates (100%). The subtype vacA1 was detected in 43% of the strains, while 54% showed the vacA12 genotype (Table 5.5). By conventional vacA 's' and 'm' region typing, 33% strains were s1m1, 57% s1m2, and 7% s2m2 (Table 5.3).

Association of vacA allelic types, cagA, and clinical disease:
The association of polymorphisms in different vacA regions and cagA status with clinical disease is shown in Table 5.5. A total of 87 patients out of 140 (62%) were colonized by H. pylori carrying cagA-positive and vacA1 region, 49 (35%) out of 140 patients were infected with strains carrying cagA-negative and vacA1 region (Table 5.3). 83% of H. pylori vacA1 were cagA positive and 57% of H.pylori vacA12 were cagA positive (Table 5.5).

The prevalence of cagA and vacA1 in IM patients (48%), was slightly higher than in PU patients (43%), however, this difference did not reach statistical significance (p=0.24). The vacA12 gene was present in 50% of IM patients and 48% of PU patients; however prevalence was higher in CG patients (64%).

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D- Prevalence of hrgA:

The hrgA gene was detected in only 62% (87 strains) of our H. pylori infected population. The prevalence of hrgA gene was independent of the clinical outcome and present in 31 of 50 (62%) for IM, 33 of 50 (66%) for CG, and 23 of 40 (58%) for PU patients.

There was no relationship between the cagA, vacA, and babA2 genotypes and the prevalence of the hrgA gene among H. pylori strains as shown in (Table 5.10).

Among the 140 patients studied, the cagA+ hrgA+ strains were more prevalent in IM patients (60%) compared with CG, PU patients being 28% and 43%, respectively. There was no significant difference in hrgA+vacAs1+prevalence in relation to disease outcomes among our patients. However, the relationship between the prevalence of hrgA and the triple-positive (cagAvacAs1babA2) was more pronounced among IM patients compared with CG and PU (Table 5.10).
Figure 5.1 Prevalence of *dupA* in Ireland and in other reported countries.
<table>
<thead>
<tr>
<th>Category</th>
<th>DNA/protein status</th>
<th>Major Virulence factors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain -specific genes</td>
<td>Gene positive or negative</td>
<td>- <em>cag</em> pathogenicity island (PAI)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Plasticity regions</td>
</tr>
<tr>
<td>Phase-variable genes</td>
<td>Gene positive, some produce functional proteins, other not</td>
<td>- <em>oipA, sabA, babA</em> (regulated by slipped strand mispairing)</td>
</tr>
<tr>
<td>Genes with different Structures/genotypes</td>
<td>Gene positive, but function and/or production levels of proteins different between strains</td>
<td>- <em>vacA</em> genotypes (combination of s1/s2 and m1/m2),</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- <em>cagA</em> repeat region (East Asian type and Western type)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- <em>alpAB</em> (East Asian type and Western type)</td>
</tr>
<tr>
<td>Primer name</td>
<td>Sequence (5'-3')</td>
<td>Size (bp)</td>
</tr>
<tr>
<td>-------------</td>
<td>-----------------</td>
<td>-----------</td>
</tr>
<tr>
<td><em>hrgA</em></td>
<td>TCT CGT GAA AGA GAA TTT CC</td>
<td>594</td>
</tr>
<tr>
<td></td>
<td>TAA GTG TGG GTA TAT CAA TC</td>
<td></td>
</tr>
<tr>
<td><strong>vacA i-region</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>VacF1</em></td>
<td>GTT GGG ATT GGG GGA ATG CCG</td>
<td></td>
</tr>
<tr>
<td><em>C1R</em></td>
<td>TTA ATT TAA CGC TGT TTG AAG</td>
<td>426</td>
</tr>
<tr>
<td><em>C2R</em></td>
<td>GAT CAA CGC TCT GAT TTG A</td>
<td>432</td>
</tr>
<tr>
<td><em>JHP940</em></td>
<td>GAA ATG TCC TAT ACC AAT GG</td>
<td>591</td>
</tr>
<tr>
<td></td>
<td>CCT AAG TAG TGC ATC AAG G</td>
<td></td>
</tr>
<tr>
<td><em>JHP947</em></td>
<td>GAT AAT CCT ACG CAG AAC G</td>
<td>611</td>
</tr>
<tr>
<td></td>
<td>GCT AAA GTC ATT TCG CTG TC</td>
<td></td>
</tr>
<tr>
<td><em>HP986</em></td>
<td>GCA TGT CCC AAA TCG TAG G</td>
<td>566</td>
</tr>
<tr>
<td></td>
<td>TGC ATT TCG CAT TGG CTC C</td>
<td></td>
</tr>
<tr>
<td><em>olpA</em></td>
<td>CAA GCG CTT AAC AGA TAG GC</td>
<td>450</td>
</tr>
<tr>
<td></td>
<td>AAG GCG TTT TCT GCT GAA GC</td>
<td></td>
</tr>
<tr>
<td><em>Jhp917</em></td>
<td>TGG TTT CTA CTG ACA GAG CGC</td>
<td>307</td>
</tr>
<tr>
<td></td>
<td>AAC ACG CTG ACA GGA CAA TCT CCC</td>
<td></td>
</tr>
<tr>
<td><em>Jhp918</em></td>
<td>CCT ATA TCG CTA ACG CGC GCT C</td>
<td>214</td>
</tr>
<tr>
<td></td>
<td>AAG CTG AAG CGT TTG TAA CG</td>
<td></td>
</tr>
</tbody>
</table>
Table 5.3 vacA Allelic Types and cagA Status of *H. pylori* Clinical Isolates

<table>
<thead>
<tr>
<th>Gene</th>
<th>vacA i-region type (no. of patients) (%)</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>i1</td>
<td>i2</td>
<td>i1 and i2</td>
<td>Total</td>
</tr>
<tr>
<td>vacA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>s1/m1</td>
<td></td>
<td>28(44)</td>
<td>18(25)</td>
<td>0</td>
<td>46(33)</td>
</tr>
<tr>
<td>s1/m2</td>
<td></td>
<td>36(56)</td>
<td>44(60)</td>
<td>3</td>
<td>83(57)</td>
</tr>
<tr>
<td>s2/m2</td>
<td></td>
<td>0</td>
<td>10(14)</td>
<td>1</td>
<td>10(7)</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>64(46)</td>
<td>72(52)</td>
<td>4</td>
<td>140</td>
</tr>
<tr>
<td>cagA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td></td>
<td>48(79)</td>
<td>39(52)</td>
<td>3</td>
<td>87(62)</td>
</tr>
<tr>
<td>Negative</td>
<td></td>
<td>13(21)</td>
<td>36(48)</td>
<td>1</td>
<td>49(35)</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>61</td>
<td>75</td>
<td>4</td>
<td>140</td>
</tr>
</tbody>
</table>
### Tables 5.4. Association of the presence of *H. pylori* plasticity zone genes (*JHP940, HP986, JHP947* and cagPAI) with disease outcome

<table>
<thead>
<tr>
<th>Gene</th>
<th>IM (n=50) %</th>
<th>CG (n=50) %</th>
<th>PU (n=40) %</th>
<th>Total (n=140) %</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>JHP940</em></td>
<td>1(2)</td>
<td>0</td>
<td>0</td>
<td>1(1)</td>
</tr>
<tr>
<td><em>HP986</em></td>
<td>15(30)</td>
<td>10(20)</td>
<td>4(10)</td>
<td>29(21)</td>
</tr>
<tr>
<td><em>JHP947</em></td>
<td>30(60)</td>
<td>16(32)</td>
<td>6(15)</td>
<td>52(37)</td>
</tr>
<tr>
<td>cagPAI +/<em>JHP 940</em></td>
<td>1(2)</td>
<td>0</td>
<td>0</td>
<td>1(1)</td>
</tr>
<tr>
<td>cagPAI +/<em>HP 986</em></td>
<td>15(30)</td>
<td>1(2)</td>
<td>4(10)</td>
<td>20(14)</td>
</tr>
<tr>
<td>cagPAI +/<em>JHP 947</em></td>
<td>27(54)</td>
<td>5(10)</td>
<td>6(15)</td>
<td>38(27)</td>
</tr>
</tbody>
</table>
Table 5.5. Association between *H. pylori* *vacA* s, i, and m Alleles, *cagA* gene and gastro duodenal pathologies.

<table>
<thead>
<tr>
<th>Gene</th>
<th>IM (n=50)</th>
<th>CG (n=50)</th>
<th>PU (n=40)</th>
<th>Total (n=140)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>s1vac</td>
<td>48(96)</td>
<td>39(78)</td>
<td>39(95)</td>
<td>125(89)</td>
</tr>
<tr>
<td>s2vac</td>
<td>2(4)</td>
<td>11(22)</td>
<td>1(3)</td>
<td>14(10)</td>
</tr>
<tr>
<td>l2vac</td>
<td>25(50)</td>
<td>32(64)</td>
<td>19(48)</td>
<td>76(54)</td>
</tr>
<tr>
<td>l1vac</td>
<td>24(48)</td>
<td>16(32)</td>
<td>20(50)</td>
<td>60(43)</td>
</tr>
<tr>
<td>l1vac l2vac</td>
<td>1(2)</td>
<td>2(4)</td>
<td>1(3)</td>
<td>4(3)</td>
</tr>
<tr>
<td>m2vac</td>
<td>16(32)</td>
<td>48(96)</td>
<td>26(65)</td>
<td>90(64)</td>
</tr>
<tr>
<td>m1vac</td>
<td>34(68)</td>
<td>2(4)</td>
<td>13(33)</td>
<td>49(35)</td>
</tr>
<tr>
<td>cagA</td>
<td>44(88)</td>
<td>17(34)</td>
<td>30(75)</td>
<td>91(65)</td>
</tr>
<tr>
<td>cagA1</td>
<td>24(48)</td>
<td>9(18)</td>
<td>17(43)</td>
<td>50(36)</td>
</tr>
<tr>
<td>cagA2</td>
<td>19(38)</td>
<td>9(18)</td>
<td>15(38)</td>
<td>43(31)</td>
</tr>
</tbody>
</table>

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Table 5.6.A. Signal sequence coding region of the \textit{oipA} gene of twelve different \textit{H. pylori} strains.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Sequence of the signal peptide Coding region</th>
<th>No. of CT repeats</th>
<th>Gene status</th>
</tr>
</thead>
<tbody>
<tr>
<td>30A</td>
<td>ATG\textsuperscript{1} AAAAAAGCCCTTTACTA ACTCTCTCTCTCTCGTTCTA \textsuperscript{2}</td>
<td>(6)\textsuperscript{3}</td>
<td>&quot;on&quot;</td>
</tr>
<tr>
<td>191A</td>
<td>ATG\textsuperscript{1} ATGAAAAAGCTCTTTACTA ACTCTCTCTCTCTCTA \textsuperscript{2}</td>
<td>(6)</td>
<td>&quot;on&quot;</td>
</tr>
<tr>
<td>261A</td>
<td>ATG\textsuperscript{1} ATGAAAAAGCTCTTTACTA ACTCTCTCTCTCTCTA \textsuperscript{2}</td>
<td>(6)</td>
<td>&quot;on&quot;</td>
</tr>
<tr>
<td>270A</td>
<td>ATG\textsuperscript{1} ATGAAAAAGCTCTTTACTA ACTCTCTCTCTCTCTA \textsuperscript{2}</td>
<td>(6)</td>
<td>&quot;on&quot;</td>
</tr>
<tr>
<td>517A</td>
<td>ATG\textsuperscript{1} ATGAAAAAGCTCTTTACTA ACTCTCTCTCTCTCTA \textsuperscript{2}</td>
<td>(8)</td>
<td>&quot;off&quot;</td>
</tr>
<tr>
<td>672A</td>
<td>ATG\textsuperscript{1} ATGAAAAAGCTCTTTACTA ACTCTCTCTCTCTCTA \textsuperscript{2}</td>
<td>(7)</td>
<td>&quot;off&quot;</td>
</tr>
</tbody>
</table>

1) ATG=Start codon  2) TGA=Stop codon  
3) \textit{oipA} gene signal sequences with 6, 9, 12 CT repeats are associated with functional \textit{oipA} "on". And non-functional \textit{oipA} gene "off" with 4, 5, 7, 8 CT repeats.
Table 5.6.B. Signal sequence coding region of the *oipA* gene of twelve different *H. pylori* strains.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Sequence of the signal peptide coding region</th>
<th>No. of CT repeats</th>
<th>Gene status</th>
</tr>
</thead>
<tbody>
<tr>
<td>377A</td>
<td>ATG↓AAAAAAAGCTCTCT TACTA</td>
<td>(6)^3</td>
<td>“on”</td>
</tr>
<tr>
<td></td>
<td>ACTCTCTCTCTCTCCTTGCTGGCTCCACGCTGA^2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>08/484A</td>
<td>ATG↓AAAAAAAGCTCTCTTACTA</td>
<td>(6)</td>
<td>“on”</td>
</tr>
<tr>
<td></td>
<td>ACTCTCTCTCTCTGTTGCTGGCTCCACGCTGA^2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>08/398A</td>
<td>ATG↓AAAAAAAGCTCTTCTA</td>
<td>(6)</td>
<td>“on”</td>
</tr>
<tr>
<td></td>
<td>ACTCTCTCTCTCTTGTTGCTGGCTCCACGCTGA^2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>715A</td>
<td>ATG↓AAAAAAAGCTTTTT</td>
<td>(8)</td>
<td>“off”</td>
</tr>
<tr>
<td></td>
<td>ACTCTCTCTCTCTCTGTTGCTGGCTCCACGCTGA^2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>737A</td>
<td>ATG↓AAAAAAAGCTCTTACTA</td>
<td>(9)</td>
<td>“on”</td>
</tr>
<tr>
<td></td>
<td>ACTCTCTCTCTCTCTTGTTGCTGGCTCCACGCTGA^2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>72A</td>
<td>ATG↓AAAAAAAGCTCTTACTA</td>
<td>(6)</td>
<td>“on”</td>
</tr>
<tr>
<td></td>
<td>ACTCTCTCTCTCTGTTGCTGGCTCCACGCTGA^2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1) ATG=Start codon
2) TGA=Stop codon
3) *oipA* gene signal sequences with 6, 9, 12 CT repeats are associated with functional *oipA* “on”. And non-functional *oipA* gene “off” with 4, 5, 7, 8 CT repeats.
Table 5.7: Detection of duodenal ulcer-promoting (*dupA*) gene and other pathogenicity-associated genes of *H. pylori* found in patients with IM, CG and PU.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>IM (n=50)</th>
<th>CG (n=50)</th>
<th>PU (N=40)</th>
<th>Total (n=140)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>dupA</em>+</td>
<td>21(42)</td>
<td>25(50)</td>
<td>14(35)</td>
<td>60 (43)</td>
</tr>
<tr>
<td><em>dupA</em>cagA*+</td>
<td>21</td>
<td>9</td>
<td>11</td>
<td>41 (29)</td>
</tr>
<tr>
<td><em>dupA</em>bab2*+</td>
<td>19</td>
<td>9</td>
<td>2</td>
<td>30 (21)</td>
</tr>
<tr>
<td><em>dupA</em>cagE<em>cagT</em>+</td>
<td>21</td>
<td>10</td>
<td>11</td>
<td>32 (23)</td>
</tr>
<tr>
<td><em>dupA</em>s1a*+</td>
<td>21</td>
<td>21</td>
<td>14</td>
<td>56 (40)</td>
</tr>
<tr>
<td><em>dupA</em>m1+</td>
<td>12</td>
<td>0</td>
<td>3</td>
<td>15 (11)</td>
</tr>
<tr>
<td><em>dupA</em>cagA+s1m1*+</td>
<td>11</td>
<td>0</td>
<td>3</td>
<td>14 (10)</td>
</tr>
<tr>
<td><em>dupA</em>cagA+s1m2*+</td>
<td>10</td>
<td>21</td>
<td>8</td>
<td>39 (28)</td>
</tr>
<tr>
<td><em>dupA</em>cagA+s2m2*+</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>4 (3)</td>
</tr>
<tr>
<td><em>dupA</em>cagAs1m1babA*+</td>
<td>10</td>
<td>6</td>
<td>1</td>
<td>17 (12)</td>
</tr>
</tbody>
</table>
Table 5.8 Distribution of *dupA* in different clinical groups

<table>
<thead>
<tr>
<th>Genotype</th>
<th>IM (n=50)</th>
<th>CG (n=50)</th>
<th>PU (n=40)</th>
<th>Total (n=140)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>dupA</em></td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td><em>dupA</em></td>
<td>21 (42)</td>
<td>25 (50)</td>
<td>14 (35)</td>
<td>60 (43)</td>
</tr>
<tr>
<td><em>dupA</em></td>
<td>29 (58)</td>
<td>22 (44)</td>
<td>25 (63)</td>
<td>76 (54)</td>
</tr>
<tr>
<td><em>dupA</em> + <em>dupA</em></td>
<td>0</td>
<td>3 (6)</td>
<td>1 (2.5)</td>
<td>4 (3)</td>
</tr>
</tbody>
</table>
Table 5.9 Distribution of *dupA* in relation to clinical outcome in Ireland and other reported countries.

<table>
<thead>
<tr>
<th>Region</th>
<th>No. of <em>dupA</em> positive strains/ no. of studied strains (%)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Gastritis</td>
</tr>
<tr>
<td>Ireland</td>
<td></td>
<td>25 / 50 (50)</td>
</tr>
<tr>
<td>Japan</td>
<td></td>
<td>7 / 50 (14)</td>
</tr>
<tr>
<td>Korea</td>
<td></td>
<td>2 / 30 (7%)</td>
</tr>
<tr>
<td>China</td>
<td></td>
<td>3 / 12 (25)</td>
</tr>
<tr>
<td>Colombia</td>
<td></td>
<td>15 / 40 (39)</td>
</tr>
<tr>
<td>Brazil</td>
<td></td>
<td>133 / 144 (92)</td>
</tr>
<tr>
<td>South Africa</td>
<td></td>
<td>11 / 15 (73)</td>
</tr>
<tr>
<td>Belgium</td>
<td></td>
<td>29 / 76 (38)</td>
</tr>
<tr>
<td>USA</td>
<td></td>
<td>9 / 20 (45)</td>
</tr>
<tr>
<td>India</td>
<td></td>
<td>16 / 70 (23)</td>
</tr>
<tr>
<td>Iran</td>
<td></td>
<td>34 / 68 (50)</td>
</tr>
</tbody>
</table>
Table 5.10 Comparison of the cagA status and hrgA status in *H. pylori* positive subjects.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>IM (n=50)</th>
<th>CG (n=50)</th>
<th>PU (n=40)</th>
<th>Total (n=140)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>cagA+</td>
<td>44(88)</td>
<td>17(34)</td>
<td>30(75)</td>
<td>91 (65%)</td>
</tr>
<tr>
<td>hrgA+</td>
<td>31(62)</td>
<td>33(66)</td>
<td>23(58)</td>
<td>87(62%)</td>
</tr>
<tr>
<td>cagA+hrgA+</td>
<td>30(60)</td>
<td>14(28)</td>
<td>17(43)</td>
<td>61(44%)</td>
</tr>
<tr>
<td>vacAs1hrgA+</td>
<td>31(62)</td>
<td>27(54)</td>
<td>21(53)</td>
<td>79(56%)</td>
</tr>
<tr>
<td>babA2hrgA</td>
<td>28(56)</td>
<td>9(18)</td>
<td>6(15)</td>
<td>43(31%)</td>
</tr>
<tr>
<td>cagAs1babA2hrgA</td>
<td>28(56)</td>
<td>7(14)</td>
<td>6(15)</td>
<td>41(29%)</td>
</tr>
<tr>
<td>cagPAI hrgA</td>
<td>30(60)</td>
<td>10(20)</td>
<td>16(40)</td>
<td>56(40%)</td>
</tr>
</tbody>
</table>
Table 5.11 the association of Signal Sequence Coding Region of the oipA gene and other virulence genes in (12) *H. pylori* isolated from IM patients.

<table>
<thead>
<tr>
<th>Strain No,</th>
<th>Gene status</th>
<th>cagPAI</th>
<th>cagA</th>
<th>vacA</th>
<th>babA2</th>
</tr>
</thead>
<tbody>
<tr>
<td>30A</td>
<td>&quot; on &quot;</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>s1am2</td>
</tr>
<tr>
<td>191A</td>
<td>&quot; on &quot;</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>s1am2</td>
</tr>
<tr>
<td>261A</td>
<td>&quot; on &quot;</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>s1am2</td>
</tr>
<tr>
<td>270A</td>
<td>&quot; on &quot;</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>s1bm2</td>
</tr>
<tr>
<td>517A</td>
<td>&quot; off &quot;</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>s1bm2</td>
</tr>
<tr>
<td>672A</td>
<td>&quot; off &quot;</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>s2m1</td>
</tr>
<tr>
<td>377A</td>
<td>&quot; on &quot;</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>s1am1</td>
</tr>
<tr>
<td>08/484A</td>
<td>&quot; on &quot;</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>s1am2</td>
</tr>
<tr>
<td>08/398A</td>
<td>&quot; on &quot;</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>s1am2</td>
</tr>
<tr>
<td>715A</td>
<td>&quot; off &quot;</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>s1bm2</td>
</tr>
<tr>
<td>737A</td>
<td>&quot; on &quot;</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>s1am1</td>
</tr>
<tr>
<td>72A</td>
<td>&quot; on &quot;</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>s1am1</td>
</tr>
</tbody>
</table>
5.3 Discussion

Many putative virulence genes of *H. pylori* have been reported to determine clinical outcomes among infected populations. Currently these virulence genes are classified into three main categories (Yamaoka, 2008) and are summarized in Table 5.1. Although a number of putative virulence factors for *H. pylori* had been reported including the *cag PAI*, vacA, iceA, and babA, their presence has typically been associated with an increased risk of both gastric cancer and peptic ulcer disease (Basso *et al*., 2008; Bolek *et al*., 2007; Graham and Yamaoka, 1998; Martins *et al*., 2005; Prinz *et al*., 2001; van Doorn *et al*., 1998; Yamaoka *et al*., 2002). These known bacterial markers undoubtedly contribute to the genesis of *H. pylori* associated diseases, but fail to distinguish between the gastro duodenal pathologies. Thus studies focusing on new putative bacterial virulence determinants are still desirable.

The plasticity zone is a hypervariable region that contains almost half of the strain-specific genes of *H. pylori*. Although most of the genes clustered here encode putative proteins of unknown function, these genes could be important for the virulence of *H. pylori* by encoding factors that influence disease outcome. In our study, the presence of the *jhp0947*-positive *H. pylori* strains and intestinal metaplasia was positively associated (*p*<0.05). The *jhp0947* gene is homologous to *jhp0938* and *jhp253*, all known to encode for hypothetical proteins. The N-terminal site of JHP0947 is also homologous to that of JHP0477, which is part of the *cagPAI* and has been identified as an important building block for the type IV secretion system encoded by the *cagPAI* (Tanaka *et al*., 2003). These observations suggest that *jhp0947* can be considered as a marker for *H. pylori* -related precancerous lesions such as intestinal metaplasia, and it might be considered as
a novel virulence marker for virulent *H. pylori*-related gastric diseases. This is in accordance with other studies that reported the association between jhp0947 and clinical diseases (Santos *et al.*, 2003; Occhialini *et al.*, 2000). In contrast to the other studies (Occhialini *et al.*, 2000; Santos *et al.*, 2003), the prevalence of the *jhp940* gene was found in only 1% of the strains, while low frequency was also observed for *HP986* gene in our strains (Table 5.4). These discordant results may be explained by differences between the plasticity regions of *H. pylori* strains isolated in distinct geographic areas. There is also considerable geographic diversity among *H. pylori* gene sequences, and therefore failure to obtain a PCR product with a single pair of PCR primers does not necessarily mean that a particular gene is absent. In our study, an association between the presence of the *jhp0947* gene and intestinal metaplasia was observed. Therefore the *jhp0947* gene as well as the *cagPAI* may be implicated in the development of intestinal metaplasia in our population. Functional analysis of the *jhp0947* gene potentially identified as a novel pathogenicity marker will provide insight into its role in the virulence of *H. pylori* related gastroduodenal diseases.

Genetic analysis of *H. pylori* in East Asia and South America has shown that *dupA* gene located in the plasticity region of *H. pylori* genome is significantly associated with an increased risk of DU and a reduced risk of gastric atrophy, intestinal metaplasia, and gastric cancer (Lu *et al.*, 2005). Our aim was to find the most probable *H. pylori* genotype associated with gastric pathologies. The presence or absence of *dupA* gene of *H. pylori* in different clinical groups is displayed in Table 5.8.
Our study confirms that the presence of \textit{jhp0917} and \textit{jhp0918} is linked to each other and constitutes the complete \textit{dupA} gene, which is in accordance with the previous studies (Lu \textit{et al.}, 2005; Douraghi \textit{et al.}, 2008; Gomes \textit{et al.}, 2008).

Our results also showed that \textit{dupA} was not associated with any disease category in our population. This is in agreement with the other studies from European populations (Argent \textit{et al.}, 2007), and in accordance with some studies from Asian and South American populations (Gomes \textit{et al.}, 2008; Douraghi \textit{et al.}, 2008 Pacheco \textit{et al.}, 2008). However some aspects were in contrast to other studies (Arachchi \textit{et al.}, 2007; Lu \textit{et al.}, 2005). Overall our data show that the prevalence of \textit{dupA} in our limited number of PU patients was lower at 23\% compared to 42 and 35\% of CG, IM patients, respectively (Table 5.7). The variations in the prevalence of \textit{dupA} in different countries may be partly due to the distinct dyspeptic population studied and the geographical heterogeneity of \textit{H. pylori} strains. The distribution of \textit{dupA} in relation to clinical outcomes in Ireland was comparable to that in other countries is shown in Table 5.9.

An OMP of \textit{H. pylori} known as HopH (designated HP0638 or OipA) may contribute to the development of gastric disease (Yamaoka \textit{et al.}, 2002). Nearly all \textit{H. pylori} strains contain \textit{oipA} alleles, but there is variation among strains in the expression of the \textit{oipA} protein because of slipped-strand mispairing and frameshift mutation in the 5' region of \textit{oipA} gene. \textit{H. pylori} strains that contain an in-frame \textit{oipA} allele typically contain other markers of virulence, including \textit{cagA}, \textit{vacA1} and \textit{babA2} (Ando \textit{et al.}, 2002; Dossumbekova \textit{et al.}, 2006 Lehours \textit{et al.}, 2004; Yamaoka \textit{et al.}, 2002; Zambon \textit{et al.}, 2003). In our results we found that all of our \textit{H. pylori} strains were \textit{oipA}-positive. With the small number of \textit{H. pylori} strains from IM
patients our results show that the functional status of \textit{oipA} gene in the 12 \textit{H. pylori} strains (75\%) were \textit{oipA} "on" status, and closely related to other \textit{cagA}, \textit{cagPAI}, \textit{vacAs1am2}, and \textit{babA2}. This result is in accordance with the other studies (Dossumbekova \textit{et al.}, 2006; Lehours \textit{et al.}, 2004; Yamaoka \textit{et al.}, 2002).

Although there is agreement that OipA is involved in the attachment of the \textit{H. pylori} to the host cells (Yamaoka \textit{et al.}, 2004; Dossumbekova \textit{et al.}, 2006), it was demonstrated in vitro that \textit{oipA} has the ability to induce IL-8 from gastric epithelial cells (Yamaoka \textit{et al.}, 2000). The role of \textit{oipA} in stimulating IL-8 expression was reported to be distinct from that of the \textit{cagPAI} in this phenomenon. Thus, \textit{cag-} positive strains expressing \textit{oipA} stimulated higher levels of IL-8 production than did \textit{cag-}positive; \textit{oipA}-negative strains (Yamaoka \textit{et al.}, 2000). Recently another study suggested that OipA modulates cell surface receptor activation and provides a driving force that initiates signal transduction pathways leading to changes in motility, cytoskeletal reorganization and elongation of gastric epithelial cells (Fazal \textit{et al.}, 2008).

Our study evaluates the presence of \textit{Helicobacter pylori} restriction endonuclease-replacing gene (\textit{hrgA}) among subjects with various gastric disorders. These results show presence of \textit{hrgA} in 87 (62\%) of \textit{H. pylori} strains and the prevalence of \textit{hrgA} gene in each group of the patients was very close to each other (Table 5.10).

The combination of a number of bacterial virulence determinants such as the \textit{cagPAI}, \textit{vacA}, and \textit{babA2} genes in combination with \textit{hrgA} gene had no significant association with respect to the clinical outcomes, the main reason being the highly plastic genome of \textit{H. pylori} (Ladeira \textit{et al.}, 2004). Other studies have reported a
similar lack of association between the hrgA and clinical outcome or to other important putative virulence factors (Lu et al., 2005A; Tiwari et al., 2008).

One of the main virulence determinants of H. pylori is the vacuolating cytotoxin, VacA (Cover and Blanke, 2005). The vacA gene encoding VacA is detectable in all strains, being polymorphic, with different types encoding VacA with different levels of vacuolating cytotoxin activity. The vacA s1/m1 and s1/m2 strains have been associated with PU, and s1/m1 strains have been associated with gastric cancer (Atherton et al., 1997; Rhead et al., 2007). A new polymorphic region in vacA, termed as the intermediate (i) region has been described in some studies (Rhead et al., 2007; Basso et al., 2008). Using PCR technique to study the association of both the new and the established H. pylori virulence markers (vacA s, m, i region), our results showed the vacA i region genotype was detected in all of the H. pylori isolates. The vacAl1 was present in 43% of the strains, and 54% showed the vacAl2. These results also showed that 44% of s1/m1vac alleles were type i1; all s2/m2 alleles were i2. The s1/m2 alleles were i1 and i2 (56% and 60% respectively). These findings are in agreement with some previous studies (Rhead et al., 2007; Basso et al., 2008). In our H. pylori strains the subtype vacA s1, i1, and m1 were all significantly associated with intestinal metaplasia, confirming that these vacA alleles are risk factors for the development of the gastric diseases as reported in previous studies (Rhead et al., 2007; Basso et al., 2008). Our study also showed that vacAl1 separately or in combination with cagA increases the risk of the H. pylori associated diseases, including peptic ulcer and the precancerous lesions (Table 5.5). However some studies have shown that vacAl genotype was not associated with a particular gastric pathology (Ogiwara et al., 2008; Sheu et al., 2009).
In conclusion, we found that in this Irish population, carriage of oipA"on"/vacA1l1m1 genotypes in the presence or absence of cagA conferred significantly increased risk of peptic ulcer disease and precancerous lesions.

Our results also suggest that jhp0947 can be considered as a marker for H. pylori related precancerous lesions such as intestinal metaplasia and it might be considered as a novel virulence marker of virulent H. pylori related gastric diseases. Our study demonstrates that the presence of dupA, hrgA genes is not an ideal surrogate marker for identifying individuals with higher risk of developing overt gastro duodenal diseases such as gastric cancer.
CHAPTER 6
COMPARATIVE PROTEOMICS AND IMMUNOPROTEOMICS
OF HELICOBACTER PYLORI CLINICAL ISOLATES
6.1. Introduction

*H. pylori* is highly adapted to the gastric mucosa, with a unique array of features that permit entry into the mucus, swimming and spatial orientation in the mucus, attachment to epithelial cells, evasion of the immune response, and, as a result, persistent colonization and transmission.

*H. pylori* are now recognized as one of the most common pathogens afflicting humans. Epidemiological studies demonstrate that *H. pylori* infection is one of the most distributed infections in the world. Infectivity of the adult population in the world varies from 30% in Western Europe and United States, to about 80% of the population in many developing countries (Mini *et al.*, 2006).

The genomes of four strains of *H. pylori* (26695, J99, HPAG1, and G27) have been sequenced (Tomb *et al.*, 1997; Alm *et al.*, 1999; Oh *et al.*, 2006; Baltrus *et al.*, 2009). The genome of *H. pylori* (1.65 million bp) contains about 1600 genes. These genes codes for about 1500 proteins (Tomb *et al.*, 1997, Alm *et al.*, 1999; Oh *et al.*, 2006; Baltrus *et al.*, 2009).

Among the most remarkable findings of two *H. pylori* genome-sequencing projects (26695 and J99) were the discovery of a large family of 32 related outer-membrane proteins (Hop proteins) that includes most known *H. pylori* adhesins and the discovery of many genes that can be switched on and off by slipped-strand mispairing-mediated mutagenesis. Proteins encoded by such phase-variable genes include enzymes that modify the antigenic structure of surface molecules, control the entry of foreign DNA into the bacteria, and influence bacterial motility. The genome of *H. pylori* changes continuously during chronic colonization of an individual host by importing small pieces of foreign DNA from other *H. pylori*
strains during persistent or transient mixed infections (Falush et al., 2001, Suerbaum et al., 1998).

DNA typing has established that _H. pylori_ is extremely diverse as a species (Kivi et al., 2007), and the varied outcomes of infection reflect differences in bacterial genotype, genetic predisposition, and immunological factors in the human host (Maeda and Mentis, 2007; Kandulski et al., 2008).

_H. pylori_ causes continuous gastric inflammation in virtually all infected persons (Dooley et al., 1989) This inflammatory response initially consists of the recruitment of neutrophils, followed by T and B lymphocytes, plasma cells, and macrophages, along with epithelial-cell damage (Goodwin et al., 1986; Suerbaum and Michetti, 2002; Andersen, 2007).

Circulating antibodies reflect a molecular imprint of antigens that are specifically related to autoimmune diseases, cancer or invading pathogens. Importantly, serum antibodies are useful clinical markers as they carry diagnostic information from all around the human body. Moreover, the amplification cascade governed by the humoral immune system causes a surplus of circulating antibodies after appearance of the corresponding (low abundance) antigen. In combination with the fact that antibodies are highly stable compared to many other serum proteins, they seem ideal to be implemented in clinical diagnostic assays for the detection of antigen-associated diseases (Tjalsma et al., 2008).

Immunoproteomics is a relatively new concept in the field of proteomics. This expression was first introduced by Jungblut (Jungblut, 2001). The key issue in immunoproteomics is to visualise what is seen by the immune system. It is a novel strategy combining standard proteomics with immunological screening; it is
currently the method of choice for identification of new antigens of diagnostic and protective values. Immunoproteomics takes advantage of the fact that higher organisms contain a highly sophisticated immune system that has the ability to distinguish normal (self) from abnormal (non self) proteins. In general, proteins that are regarded as non self by the immune system are called antigens (antibody generators) and can be defined as proteins that bind to immune receptors and elicit an immune response (Alberts et al., 2002).

There are numerous potential diagnostic applications for Immunoproteomics-based assays to diagnose and monitor diseases that are related to antigenic proteins. Some of the applications are:

1-Monitoring vaccine efficiency
Diseases caused by bacteria and viruses can be prevented by vaccines, which generate neutralizing antibodies against surface antigens of the relevant pathogen. Therefore, it is very important to identify highly efficient immunogens for immune prevention and monitor vaccination strategies (Chen et al., 2004; Davies et al., 2007).

2- Monitoring antigenicity of therapeutic drugs
Immunogenicity is a significant problem associated with protein therapeutics (de Groot and Moise, 2007). Due to artificial production processes, recombinant therapeutics is often not completely identical to their human native counterparts. In some cases, this therapeutics elicits an immune response that interferes with drug efficacy. In principle, immunoproteomic approaches could be used to define the patient immune response against a panel of related (protein) drugs to aid in selective drug application in order to optimize individualized treatment.
3- Diagnosis and prognosis of autoimmune diseases

Autoimmune diseases such as rheumatoid arthritis (RA) and autoimmune type I diabetes, arise from erroneous activation of the immune system, resulting in the attack of self-proteins within the human body. In addition, autoimmune-like diseases can occur during blood transfusions or pregnancy when antirhesus antibodies are formed that target the red blood cells from the donor or foetus, respectively (Avent et al., 2006). Moreover, an autoimmune reaction towards placental components has been implicated in the development of preeclampsia (Sargent et al., 2003), which is a life-threatening situation for pregnant women. Furthermore, allergic diseases can be characterized by IgE antibodies targeting allergens. In all these cases, immunoproteomic diagnosis and disease monitoring is feasible (Harwanegg and Hiller, 2006). Altogether, these examples show that immunoproteomics holds great promise to aid in diagnosis, prognosis and monitoring of autoimmune (like) diseases (Tjalsma et al., 2008).

4- Early detection of cancer

Many cancer patients produce antibodies against antigens that are specifically expressed by malignant cells, which is indicative that immune response is a valuable source of diagnostic and prognostic information. The use of antibodies against several tumour-specific antigens has been described, exemplified by p53, NY-ESO-1, and Muc1 (Soussi, 2000; Hermsen et al., 2007; Caron et al., 2007). This cancer-related immune response to self proteins is driven by tumour-induced molecular alterations, such as mutation, misfolding, (over) production, aberrant degradation or glycosylation that renders these proteins immunogenic. However, a tumour protein that is normally only expressed during embryonic development, have antigenic properties. Characterization of antibodies against panels of tumour
antigens has proven to be more informative than detection of antibodies against individual specificities (Shi et al., 2005). Importantly, it has been observed that antibody responses to p53 were diagnostic for asymptomatic liver tumours in individuals that were exposed to toxic compound (Trivers et al., 1995). Strikingly these antibody responses could even predate clinical diagnosis of oesophageal cancer (Cawley et al., 1998). Using a gel-free LC-MS/MS approach to identify a panel of ovarian tumour antigens to which antibodies were present in patients with early stage disease (Philip et al., 2007).

5-Diagnosis of infection

The main task of the adaptive immune system is to recognize invading antigens and their matching pathogens and subsequently attack and destroy them. Therefore the antipathogen antibodies are good markers for the diagnosis of parasitic, fungal, microbial and viral infection (Coleman et al., 2007). The immunoproteomics-based assay was used to diagnose Lyme disease, and also provide valuable information on disease progression (Nowalk et al., 2006).

The accumulation of genomic information, combined with advances in protein separation and identification techniques (Humphery-Smith et al., 1997), has made it possible to identify the entire protein complement expressed in an organism, facilitating the search for virulent strains of pathogenic bacteria and disease-specific markers (Cash, 2000). The genetic diversity of H. pylori results in phenotypic variations, including the alteration of gene expression, which can influence the outcome of an infection (Alm and Trust, 1999). A variety of H. pylori components have been investigated at genetic or serologic levels for the

Although several proteins have been reported to be associated with severe symptoms of gastric disease, most of these require further evaluation of expression levels in order to clarify their relationship with gastric disease patterns. A proteomic approach provides simultaneous, multifaceted data for analyzing the expression levels of bacterial proteins.

The Aims:-

1- To compare the proteomic profiles of Irish *H. pylori* strains isolated from *H. pylori* -infected adult patients with gastric diseases.

2- To identify the different proteins of *H. pylori* in three groups of patients with Chronic Gastritis (CG), Intestinal Metaplasia (IM), and Peptic Ulcer (PU).

3- To investigate potential associations between specific immune responses and manifestations of disease.

4- To evaluate the application of Immunoproteomics methods in research on *H. pylori*.
Figure 6.1. An outline of a general strategy to perform proteomics

- Solubilise proteins from cells/tissue
- 2D-gel electrophoresis
- Image analysis
- Isolation of spots of interest
- Trypsin digestion of protein
- MALDI-TOF mass spectrometry of tryptic fragments
- Identification of proteins
6.2 Results

The comparative proteomic and immunoproteomics study was performed using six *H. pylori* clinical isolates with different genotypes (Table 6.1).

The differences between some of the *H. pylori* strains were characterized in terms of the protein composition of the strains in a preliminary manner by 2D SDS-PAGE. Some of the major proteins of *H. pylori* that may be associated with gastric disease as determined by analysis of the immunoreactivity of patient sera to these antigens are shown in Table 6.2. However, it must be emphasised here that the identification of these proteins is based on comparing their relative position in the 2D profiles generated in this study with a published 2-DE reference map of *Helicobacter pylori* strain 26695 (www.mpiib-berlin.mpg.de), and not by mass spectrometric analysis.

The proteins from lysed cell pellets of six *H. pylori* clinical isolates were separated on a series of 2D gels run in parallel with a pH gradient of pH 3 to pH 10 for the IEF dimension. The sliver - stained gels revealed quite similar patterns between the *H. pylori* isolates from three groups of patients, however differences were observed in the presence/absence and/or intensity of individual proteins, most notably in clusters of spots at approximately 80, 45, and 30 kDa (Figures 6.2, 6.3, and 6.4).

The pattern of expression of the chaperonin (GroEL) protein (spot No.2) was different between the three groups of patients; it was higher in Chronic Gastritis 2-DE gel than by those from intestinal metaplasia, peptic ulcer patients (Figure 6.2).
The proteins numbered 9 and 10 which represent the adhesion-thiol peroxidase (TagD) and Cag pathogenicity Island (Cag 26) proteins were only visible in the isolates obtained from patients with peptic ulcer (Figure 6.3), but they were not visible in \textit{H. pylori} strains from intestinal metaplasia, chronic gastritis patients (Figure 6.2, 6.4). Whether this is due to insufficient biomass being loaded on the gel or due to lack of expression remains to be determined.

To analyse the immune response of the three patient groups to the antigens from \textit{H. pylori}, the proteins from the 2D-gels were transferred to polyvinylene difluoride (PVDF) membranes by semidry blotting and incubated with a positive serum pool or a negative serum pool. Western blot data revealed that at least 10 groups of spots were recognized by antibodies in the infected patient serum pool (Figures 6.5, 6.6, and 6.7), whereas the pooled sera from three uninfected persons that was used as a negative control did not exhibit any immunoreactivity.

**Comparison of antigenic reactivity patterns for strains probed with the same serum:**

While the protein profiles of various \textit{H. pylori} strains obtained by analysis of 2D-gels were similar, the Western blot profiles showed subtle differences, when probed with serum from the different groups of patients. The six patients were found to be seropositive for the four house-keeping antigens (UreB, GroEL, EF-TU, and FlaA). While all sera samples contained antibodies to the \textit{H. pylori} proteins, the spectrum of responses varied among patients. For example, the serum obtained from a patient with intestinal metaplasia demonstrated greater reactivity with the homologous strain that appeared to be distinct from that found when compared with those from peptic ulcer and chronic gastritis (Figure 6.5, 6.6, and 6.7).
Table 6.1: *H. pylori* clinical strains used

<table>
<thead>
<tr>
<th>Strain No.</th>
<th>Pathology</th>
<th>cagA</th>
<th>vacA</th>
<th>babA2</th>
</tr>
</thead>
<tbody>
<tr>
<td>08/168A</td>
<td>Chronic Gastritis</td>
<td>+</td>
<td>s1am2</td>
<td>-</td>
</tr>
<tr>
<td>496</td>
<td>Chronic Gastritis</td>
<td>+</td>
<td>s1am2</td>
<td>+</td>
</tr>
<tr>
<td>376</td>
<td>peptic Ulcer</td>
<td>+</td>
<td>s1am2</td>
<td>+</td>
</tr>
<tr>
<td>08/377</td>
<td>Peptic Ulcer</td>
<td>+</td>
<td>s1am1</td>
<td>-</td>
</tr>
<tr>
<td>715</td>
<td>Intestinal Metaplasia</td>
<td>+</td>
<td>s1m2</td>
<td>+</td>
</tr>
<tr>
<td>270</td>
<td>Intestinal Metaplasia</td>
<td>+</td>
<td>s1m2</td>
<td>+</td>
</tr>
</tbody>
</table>
Table 6.2. The corresponding major spots of the six *H. pylori* clinical isolates. The designation and protein name according to proteome 2DE-PAGE Database of the reference strain *H. pylori* 26695 (www.mpiib-berlin.mpg.de/2D-PAGE).

<table>
<thead>
<tr>
<th>Spot No.</th>
<th>Designation</th>
<th>Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HP0072</td>
<td>Urease β-subunit (UreB)</td>
</tr>
<tr>
<td>2</td>
<td>HP0010</td>
<td>Chaperonin (GroEL)</td>
</tr>
<tr>
<td>3</td>
<td>HP1205</td>
<td>Elongation factor (EF-Tu)</td>
</tr>
<tr>
<td>4</td>
<td>HP0601</td>
<td>Flagellin (FlaA)</td>
</tr>
<tr>
<td>5</td>
<td>HP0795</td>
<td>Trigger factor (Tig)</td>
</tr>
<tr>
<td>6</td>
<td>HP1561</td>
<td>Iron –binding protein (CeuE)</td>
</tr>
<tr>
<td>7</td>
<td>HP1563</td>
<td>Alkyl hydroperoxide reductase (TsaA)</td>
</tr>
<tr>
<td>8</td>
<td>HP1161</td>
<td>Flavodoxin (FIdA)</td>
</tr>
<tr>
<td>9</td>
<td>HP0390</td>
<td>Adhesin-thiol peroxidise (TagD)</td>
</tr>
<tr>
<td>10</td>
<td>HP0547</td>
<td>Cag pathogenicity island protein (Cag26)</td>
</tr>
</tbody>
</table>
Figure 6.2: two-dimension gel of *Helicobacter pylori* clinical isolates 08/377 (Peptic Ulcer) stained with Silver nitrate. The numbers (1 to 10) corresponding the major spots as indicated in the Table 6.2.  
(Data from www.miipb-berlin.de/2DE)
Figure 6.3: two-dimensione gel of *Helicobacter pylori* clinical isolate 08/168A (Chronic Gastritis) stained with Sliver nitrate. Four hundred micrograms of proteins was focused in a nonlinear immobilized pH gradient strip of 3-10. After isoelectric focusing, the sample was run on a 12% SDS-PAGE gel and subsequently stained with Coomassie blue and silver nitrate. The numbers (1 to 10) corresponding the major spots as indicated in the Table 6.2. (Data from www.miipb-berlin.de/2DE)
Figure 6.4: two-dimension gel of *Helicobacter pylori* clinical isolates 715 (Intestinal Metaplasia) stained with Silver nitrate. The numbers (1 to 10) corresponding the major spots as indicated in the Table 6.2. (Data from www.miipb-berlin.de/2DE).
Figure 6.5  *H. pylori* antigens detected by immunostaining on two-DE blots with serum from patient with Chronic Gastritis. The white arrow indicate the highly immunoreactive region of the patient serum with spots numbers 1, 2, 3, 4, 5 which represent UreB, GroEL, EF-TU, FlaA, and Tig antigens respectively.
Figure 6.6. *H. pylori* antigens detected by immunostaining on two-DE blots with serum from patient with Peptic Ulcer. The two arrows indicate the highly immunoreactive regions of the patient serum with spots numbers 1, 2, 3, 4, 5 which represent UreB, GroEL, EF-TU, FlaA, and Tig antigens respectively (yellow arrow), the white arrow represent the CeuE antigen with the patient serum.
Figure 6.7. *H. pylori* antigens detected by immunostaining on two-DE blots with serum from patient with Intestinal Metaplasia. The two arrows indicate the highly immunoreactive regions of the patient serum with spots numbers 1, 2, 3, 4, 5 which represent UreB, GroEL, EF-TU, FlaA, and Tig antigens respectively (yellow arrow), the white arrow represent the Cag26 antigen with the patient serum.
6.3 Discussion

*H. pylori* is a widespread human gastric pathogen which affects 60% of the worldwide population. It is accepted as the most important agent of gastritis in humans, as well as a risk factor for peptic ulcer disease and gastric carcinoma (Godoy et al., 2007). The variety of pathologies related to *H. pylori* is a consequence of host physiological and immunological factors, bacterial genotypes variability and host/bacterium geographical localization (Mini et al., 2006). One aim of the present study was to compare the 2-DE proteomic maps of *H. pylori* in order to characterize the protein patterns of six clinical isolates derived from intestinal metaplasia, peptic ulcer, and chronic gastritis patients.

The completion of the four *H. pylori* genome sequences has permitted proteomic approaches to be applied for identification of *H. pylori* proteins (Jungblut et al., 200; Bumann et al., 2001; Bjorkholm et al., 2001; Sun et al., 2008), and protein–protein interactions (Rain et al., 2001). More than 1800 *H. pylori* protein species have thus far been resolved by 2-DE (www.mpiib-berlin.mpg.de/2D-PAGE). These data have subsequently spawned further proteomic studies including *H. pylori* antigen detection (Mc Atee et al., 1998; Nilsson et al., 2000; Haas et al., 2002; Krah et al., 2004; Lin et al., 2006; Sun et al., 2008), identification of cell surface proteins (Sabarth et al., 2002; Backert et al., 2005), and pH-dependent protein induction (Sionczewski et al., 2000). In addition, 2-DE was applied to study post-translational modifications, such as the tyrosine phosphorylation of CagA (Backert et al., 2001), the processing of CagA (Moese et al., 2001), and the processing of VacA (Bumann et al., 2002).
The results shown here demonstrate a strongly conserved pattern of protein expression across the six strains studied. While in this study we did not perform protein identification by mass spectrometry (MS) on corresponding protein spots from the *H. pylori* strains studied, the consistency of the staining pattern across the six *H. pylori* strains indicated that a similar consistency of protein identities might reasonably be inferred (Figures 6.2, 6.3, 6.4).

The study found a quite heterogeneous antigenic pattern was recognised by the patient’s sera. The sera from intestinal metaplasia patients recognised more antigens on 2D immunoblots of *H. pylori* compared to sera from peptic ulcer patients. Similarly, the sera from peptic ulcer patients were richer in antibodies compared to chronic gastritis patients (Figure 6.5, 6.6, and 6.7). This result is consistent with other studies that reported gastric cancer patient sera provided stronger immunoreactivity directed against the *H. pylori* proteins (Mini et al., 2006; Lin et al., 2006), and with other studies reporting that ulcer patients have more anti-*H. pylori* antibodies than gastritis patients (Haas et al., 2002).

In this study the total cell proteins were extracted from six clinical strains. The immunoblotted protein from the 2-DE gels was performed using pooled antisera from patients who had been diagnosed with *H. pylori* infection and a control serum pool from individuals diagnosed as being free from *H. pylori* infection. The results showed the identification of an immunodominant region, bounded on the gels by the pl range 4 -7, and molecular weight range 20 -80 kDa, a finding that is consistent with those of previously published studies (Mc Atee et al., 1998; Jungblut et al., 2000; Kimmel et al., 2000; Lock et al., 2002; Lin et al., 2006; sun et al., 2008).
Although the proteome technique proved to be a useful approach for the identification of immunogenic proteins, the detection of marker antigens that correlated with a particular gastro duodenal pathology was not observed in this study. Undoubtedly this could be optimised to improve resolution and the number of antigens detected. For example, in preliminary studies designed to identify the optimum amount of serum to use in the immunoblotting, considerably more antigens were recognised by the patient serum on 1D immunoblots compared with the 2D immunoblots. The reasons for this are not clear at present however it is possible that additional improvements to the blotting protocol would help this issue. Furthermore, certain factors contributing to *H. pylori* virulence may not be expressed under in vitro culture conditions, but their expression may depend on certain in vivo stimuli, for example cell-to-cell contact. Also, nothing is known about the way in which the course of the chronic *H. pylori* infection influences the immune response. Therefore the variability in response to *H. pylori* antigens found here suggests that it would be worthwhile conducting a larger prospective study, incorporating proteomic approaches to characterize changes in serum responses following *H. pylori* infection.

The results showed that spot number (2) which represents the Chaperonin (GroEL) protein was different between the three groups of patients, and similar results were described by other previous studies who reported that the pattern of expression of GroEL was different in gastro duodenal diseases (Krah *et al.*, 2003; Pereira *et al.*, 2006; Lin *et al.*, 2006).
Our results showed that *H. pylori* clinical isolates share similarity in the majority of the protein spots; however some proteins varied in expression level. Spots corresponding to the adhesion-thiol peroxidase (TagD), the Cag pathogenicity Island (Cag 26) proteins were visualized only on the proteome map of peptic ulcer 2-DE gel (Figure 6.3), and not in the other groups of patients.

This study demonstrates that the distinct patterns of protein expression in each sample suggest that *H. pylori* clinical isolates are able to express proteins distinctly. However, further studies using mass spectrometry technology will provide identification of other bacterial proteins, to confirm whether the gastro duodenal diseases are related to different patterns of *H. pylori* protein expression.

In conclusion, it may be suggested that the typing method based on the proteomic procedure is informative, even without protein identification by means of mass spectrometry. However, considerable further work is required to establish the significance of these preliminary observations.
CHAPTER 7
GENERAL DISCUSSION AND FUTURE DIRECTIONS
*H. pylori* has been the subject of intense investigation since its culture from a gastric biopsy during the last two decades. Stomach infection with *H. pylori* is the most common infectious disease of humans. The severe pathological consequences of this infection include gastric and duodenal ulcer disease, the development of gastric atrophy, intestinal metaplasia and gastric carcinoma. There are inter-individual differences in the extent of gastric inflammation among *H. pylori* infected patients, and clinical consequences develop in only a small subgroup. Bacterial virulence factors, such as the *cag* pathogenicity island (*cagPAI*), the vacuolating cytotoxin (*vacA*) and the blood group antigen-binding adhesion (*BabA*) are associated with enhanced inflammation and cancer development (Atherton *et al.*, 1995; Bartchewsky *et al.*, 2009; Censini *et al.*, 1996; Gerhard *et al.*, 1999; Prinz *et al.*, 2001; Olivares *et al.*, 2006; Serrano *et al.*, 2007). The study described with this thesis investigated the prevalence of the established and novel virulence factors of *H. pylori* using PCR and Nested PCR in patients with different gastro duodenal diseases. Since *H. pylori* may no longer be detected in patients with gastric cancer, the study of *H. pylori* infected noncancerous patients offers the golden opportunity to examine the potential of the bacterial virulence factors in the gastric carcinogenesis process. Intestinal Metaplasia was chosen as the histological category for comparison because it is well known that individuals who have gastric intestinal metaplasia have a definite increase in cancer risk (Correa, 1992). The vast majority of *H. pylori* in colonized hosts is free living; however, 15%-20% bind specifically to gastric epithelial cells. The adherence of *H. pylori* to the host gastric mucosa is required for prolonged persistence in the stomach and for induction of injury and disease (Gerhard *et al.*, 1999; Mahdavi *et al.*, 2002). *H. pylori* adherence is likely to be an important determinant virulence factor in vivo since infection is associated with a high degree of species specific
tissue tropism (Logan et al., 1998; Zhang et al., 2002). It has been reported that *H. pylori* strains have either a functional or non-functional *olpA* gene. Functional status of the *olpA* is regulated by a slipped strand repair mechanism based on the number of CT dinucleotide repeats in the 5' region of the gene (Yamaoka et al., 2000). Irish *H. pylori* strains have functional or non-functional *olpA* gene, depending on the number of CT dinucleotide repeats in the coding region of the gene. The *olpA* "on" strains were simultaneously *cagA* positive. Furthermore, *olpA* "on" strains were also associated with presence of the *vacAs1*, and *babA2* genes. These findings of the study are in agreement with some previous studies (Ando et al., 2002; Dossumbekova et al., 2006; Franco et al., 2008; Lehours et al., 2004; Yamaoka et al., 2002; Zambon et al., 2003). *H. pylori* induced gastric inflammatory reaction does not depend on a single virulence factor, but probably results from the synergistic effect of multiple virulence factors, which work together in a complex way, causing damage to the host, this was more clearly demonstrated with intestinal metaplasia and peptic ulcer patients. Future studies on a large population of adults will help to establish such correlation.

In this thesis all paired (antrum and corpus) *H. pylori* isolates examined displayed identical allelic combinations at eight different loci. To determine the relatedness of pairs of isolates RAPD-PCR was performed. This fingerprinting technique demonstrated that the majority of the pairs shared identical profiles. Of the six pairs that displayed identical profiles, one pair had differences in the *cagA* and had identical genotypes at *cagE*, *cagT*, *babA2*, and *dupA*. This indicates that assessment of the relatedness of *H. pylori* strains residing in the same host by specific genotyping at multiple loci alone will not always be reliable (Owen and Xerry, 2003; Han et al., 2003). The minor differences observed in fingerprint
profiles with paired isolates may be due to insertions, deletions or repeated sequences that commonly occur within \textit{H. pylori} genomes (Taddei \textit{et al.}, 1997). It was suggested that to identify strain specific genes, instead of RAPD-PCR, the PCR sequencing techniques are more reliable and reproducible (Han \textit{et al.}, 2003).

The prevalence of mixed infection in an Irish population is 9%. The detection of multiple genotypes implies the presence of multiple strains in a clinical sample. The risk of coinfection or superinfection with multiple strains may be higher in countries with high prevalence of \textit{H. pylori} infection than in those with a low prevalence (Ghose \textit{et al.}, 2005; Momynaliev \textit{et al.}, 2003; Morales-Espinosa \textit{et al.}, 1999; Sheu \textit{et al.}, 2009; van Doorn \textit{et al.}, 1998). Multiple \textit{cagPAI} genotypes detected in this study were more prevalent in patients with CG than in those with IM or PU. Further studies will be necessary in this population to examine whether infection with multiple strains increases the risk of serious clinical outcomes such as peptic ulcer of gastric cancer or not.

Several recent studies failed to show a relationship between the \textit{H. pylori} virulence genes and clinical symptoms in several patient populations (Go and Graham 1996; Ito \textit{et al} 1997; Garcia-Campos \textit{et al.}, 2004; Quiroga \textit{et al.}, 2005; Mattar \textit{et al.}, 2005; Jafari \textit{et al.}, 2008; Zheng \textit{et al.}, 2006). The discrepancy between these reports and the results from our study may have several explanations.

The First explanation is that the patient selection is extremely important, and the study group should be sufficiently large and diverse with respect to genotypes and clinical diagnosis, such as studies did not comprise any \textit{vacA s2} or \textit{cagA}-negative patients (Ito \textit{et al} 1997; Carroll \textit{et al.}, 2003).
The Second explanation is the geographic origin of the patients may also play an important role. Some studies suggest the existence of separate bacterial lineages in different parts of the world (Campbell et al., 1997; Genta et al., 1995). Analysis of more than 700 \textit{H. pylori} strains from 24 countries showed that the vacA genotypes are not uniformly distributed over the world (van Doorn et al., 1997).

The Third explanation is that to determine the true relationship between the genotypes of \textit{H. pylori} virulence genes and clinical outcome, it is crucial to exclude patients carrying multiple strains, in many studies, it is unclear whether such cases were adequately excluded from statistical analysis (Ito et al., 1997).

The positive association between \textit{H. pylori} genotypes (vacAs1m1 / cagA+/ iceA1) and intestinal Metaplasia do not imply that patients without IM cannot be infected with high risk \textit{H. pylori} genotypes. In our study, not all patients in whom IM was diagnosed were infected with virulent genotypes of \textit{H. pylori}. Conversely, virulent genotypes cagA-positive, vacAs1m1 and iceA1 were found in patients with CG of frequencies of 17%, 7%, and 14% respectively. Several important aspects should be taken into account.

First, it is almost impossible to be completely certain of the clinical diagnosis. The role for \textit{H. pylori} in the etiology of gastric cancer has been recognized for at least a decade (IARC, 1994), and the importance of cagA as a marker of increased virulence has also long been recognized (Blaser et al., 1995). However, it has been difficult to quantify the risk of gastric cancer associated with \textit{H. pylori} infection. Most of the previous evidence that \textit{H. pylori} infection is associated with gastric adenocarcinoma or its precursors has come from seroepidemiologic studies that
detected antibodies against either *H. pylori* or the CagA protein. It is widely believed that retrospective assessment of *H. pylori* status in gastric cancer patients has low sensitivity due to a progressive loss of *H. pylori* infection as gastric atrophy develops. For this reason, prospective studies with a long follow-up period are considered to be the most reliable source of risk estimates for the gastric precancerous lesions (atrophic gastritis and Intestinal Metaplasia) and gastric cancer. Although our present study did not include gastric cancer patients, *H. pylori* DNA remains detectable in the gastric mucosa of individuals with advanced precancerous lesions. In our study, the risk of IM conferred by *H. pylori* was specific to vacAsIm1/cagA +/iceA1 strains.

Second, Links between putative virulence factors and clinical outcomes could be weakened or enhanced by host and environmental factors. This notion is consistent with recent data suggesting the importance of host factors such as smoking (Koivisto *et al.*, 2008; Jeneb *et al.*, 2008; Marakoglu *et al.*, 2008; Suzuki *et al.*, 2006), HLA status (Garza-González *et al.*, 2004; Quintero *et al.*, 2005; Hirata *et al.*, 2007), and cytokine polymorphisms (Li *et al.*, 2007; Rad *et al.*, 2004; Trejo-de la *et al.*, 2008), which may also influence the clinical outcome of *H. pylori* infection and may interact with certain bacterial virulence factors. The advent of the whole genomic sequence of both *H. pylori* and a number of hosts including the human and mouse, and the development of microarray technology have provided the ultimate research tools for understanding the relationships between pathogen and host. Microarray can be used to study a variety of aspects of a pathogen including expression profiling, comparative genomics, the bacterial response to interaction with host cells, and the host transcriptional response to *H. pylori* infection.
Third, additional bacterial virulence factors may play an important role. For example the binding to blood group antigens appears to depend on distinct polymorphisms in the \textit{babA} gene (Ilver et al., 1998), and the outer inflammatory protein (oipA), Sialic acid-binding adhesion (sabA), and \textit{hopQ} (omp27), their importance in clinical presentation, gastric inflammation have been shown in certain population. (Cao et al., 2005; de Jonge et al., 2004; Yamaoka et al., 2002; Yanai et al., 2007). Recent studies showed that strain specific genes or combination of genes in the plasticity regions may play important roles in the pathogenesis of \textit{H. pylori} associated gastro duodenal diseases (Yamaoka, 2008).

The availability of the genome sequences of \textit{H. pylori} is revolutionising research throughout the world, since about one third of the open reading frame in \textit{H. pylori} is still of unknown function. Expression profiling of these genes may help elucidate functions for these gene products. This may be particularly important as many of these products are \textit{H. pylori} specific and are likely to be directly related to the unique environment in which this organism lives and the disease manifestations it causes. An example may be the genes encoding outer membranes proteins (OMPs), the genes within the plasticity region, which have been found to be coregulated with \textit{cagA} gene. Functional analysis of these genes as novel pathogenicity markers will provide insight into its role in the virulence of \textit{H. pylori} related gastric diseases.

This thesis has demonstrated the potential of proteome technology, and the utility of PCR, nested PCR techniques, for determination of the prevalence of a number of virulence genes that might be considered as a surrogate marker of \textit{H. pylori} related diseases. Future studies, including an evaluation of the association
between these and other previously described *H. pylori* virulence factors in a large number of patients, are needed to clarify their role in the pathogenesis of this microorganism. Moreover, the study of the distribution of these genes in other populations would also be interesting to further elucidate the associations found in the present study. Given that approximately 50% of humans are infected with *Helicobacter*, that only a very small fraction of infected subjects ever develop cancer, and that eradication therapy may lead to activation of antibiotic-resistant strains of other pathogens, massive eradication is not feasible or advisable. There is therefore, a need to identify subjects at highest cancer risk because of their genetic susceptibility and being infected with *H. pylori* genotypes of greater carcinogenic potential. Thus the patients with pre-cancerous pathologies could be molecularly assessed for potential risk of carcinogenesis, and surveillance could be tailored to the degree of risk conferred.

In conclusion, infection with specific genotypes of virulent *H. pylori* is associated with intestinal metaplasia. Also this study found that:-

1. *H. pylori* genotyping on archived gastric tissue using nested PCR is reliable and good for large scale studies. It can be used on specimens from those patients who might not be available for doing serum genotyping.


3. *H. pylori* type 1 and triple-positive strains increase the risk of developing Intestinal Metaplasia.
4. Carriage of oipA"on/vacAs1/1m1 genotypes in the presence or absence of cagA confers significantly increased the risk of peptic ulcer and intestinal metaplasia.

5. The plasticity region gene jhp0947 should be considered as a marker for *H. pylori* related gastro duodenal pathologies.

6. Using a proteomics-based approach, the proteome from *H. pylori* clinical strains is quite similar; our study confirms the extensive strain variation at the protein level that *H. pylori* exhibits.
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