THE PATHOGENESIS OF *HELICOBACTOR PYLORI* ASSOCIATED DISEASES IN KURDISTAN REGION, IRAQ

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بسم الله الرحمن الرحيم

﴿قُل إنَ صَلاتي وَنُسُكي ومَحياي وَمَماتي للهِ ربِ العالمين لا شَريكَ لَهُ وبذلك
أُمِرت وأَنا أَولُ المسلمين﴾

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Declaration

Unless otherwise acknowledged, the work presented in this thesis is my own; no part has been submitted for another degree at the University of Nottingham or any other institute of learning.

Nawfal Hussein
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Abstract

Helicobacter pylori is regarded as the most important risk factor for peptic ulcer disease and gastric cancer. In Kurdistan region, northern Iraq, gastric cancer is rare (5/100,000). To investigate some possible reasons for this, the prevalence of *H. pylori* infection, gastric mucosal histopathological changes in *H. pylori* infected subjects, and virulence factor genotypes (especially *dupA*) of colonising strains were studied. The immune response to *H. pylori* infection, focusing on genes associated with T-helper (Th) and regulatory T-cell (Treg) cells, was also investigated.

It was found that 79% of 163 adults and 37% of 120 children were seropositive for *H. pylori* ($p<0.0001$). For infected people, gastric lymphocyte infiltration was more prominent in the antrum ($p=0.01$).

71% of Iraqi *H. pylori* strains were positive for *cagA* and its presence was significantly associated with peptic ulcer disease (PUD) ($p<0.01$). *cagA* genes encoding four or more tyrosine phosphorylation motifs could not be found in any of the Iraqi strains. Isolates possessing the i1 form of *vacA* were significantly associated with GU ($p<0.02$). 32% of Iraqi *H. pylori* isolates were *dupA*-positive and presence of this gene was associated with PUD ($p<0.01$).

The levels of *IFNγ*, *IL-12 p35*, *IL-10*, *IL-4* and *FOXP3* mRNA were found to be elevated in gastric mucosal samples from *H. pylori*-infected patients compared to those from *H. pylori*-negative patients (median increase 7-fold $p=0.001$; 17-fold $p=0.002$; 1320-fold $p=0.001$; 1184-fold $p=0.001$; and 3-fold $p=0.01$, respectively), indicating a predominant IL-4 and IL-10 (Th2) response. Interestingly, *IFNγ* mRNA levels were 16-fold higher in tissues taken from 17 infected smokers than found in tissues taken from 18 infected non-smokers ($p=0.009$). *IL-4* mRNA levels in tissues from 20 infected females were 40-fold higher than in tissues from 15 males ($p=0.005$).

Nucleotide sequencing of the *dupA* 3' region from 32 strains showed that *dupA* commonly had additional single base insertions or deletions that either truncated or extended the open reading frame (ORF). We have therefore classified *dupA* into two main groups: the common extended ORF within *jhp0917-19* (*dupA1)*, and *dupA* with an early stop codon to truncate the ORF (*dupA2)*. ELISA performed on supernatants from *H. pylori*-infected gastric epithelial cell lines found no significant differences in IL-8 production between strains that possessed or lacked *dupA*. In comparison to wild-type *H. pylori*, disruption of *dupA* significantly reduced IL-12, IFNγ, TNFα and IL-8 production by peripheral blood mononuclear cells (PBMCs) in 2/4 strains. For the remaining 2 strains, where gene sequencing revealed a frame shift resulting in truncated *dupA* in the wild-type, the level of these cytokines was unchanged by *dupA* mutation.

H. pylori infection is common in Kurdistan region and acquired at a young age. The low cancer rate may be partially explained by a predominant lymphocyte infiltration in the antrum rather than the corpus, which has been reported to be associated with reduced risk of gastric adenocarcinoma. An absence of the more toxic *cagA* genotype with four or more tyrosine phosphorylation motifs in the Iraqi strains, and the predominance of Th2 cytokine expression rather than a more pro-inflammatory Th1 response to *H. pylori* could also contribute to a reduced incidence of cancer. *dupA1* appears to play an important role in promoting the inflammatory response of leukocytes to *H. pylori*.

1. Introduction

1.1 Overview

Helicobacter pylori is a spiral shaped, Gram-negative rod with 5-7 flagella at one end. This bacterium is sensitive to oxygen, and requires a microaerobic atmosphere, *i.e.*, about 5% O_2 and 5-10% CO_2. Bacteria of the *Helicobacter* genus are very similar to those of the *Campylobacte*r genus. Both are motile, catalase positive and have curved cell bodies (Andersen and Wadström, 2001). *H. pylori* causes gastritis and peptic ulcers (Marshall and Warren, 1984, Marshall and Warren, 2001, Whitfield, 2003). Consequently, there has been a pivotal move in mainstream ulcer treatment from the widespread use of acid-reducing drugs (which heal ulcers but do not prevent their recurrence) to the "treatment" of gastritis and ulcers with potent antibiotics which eradicate *H. pylori* (Walsh and Peterson, 1995).

Helicobacter pylori was originally called *Campylobacter pyloridis*, but after correction to the Latin grammar the organism was renamed as *C. pylori*. In 1989, DNA sequencing data showed that the bacterium did not belong to the *Campylobacter* genus. Hence, it was placed in its own genus, *Helicobacter* (Marshall and Warren, 2001). The name “pylori” originates from the Latin word pylorus, which means gatekeeper, and refers to the pyloric valve between the stomach and duodenum.

The inside of the stomach is exposed to litres of gastric juice every day. Gastric juice is composed of digestive enzymes and concentrated hydrochloric acid. Bacteria, viruses, and other microorganisms are destroyed in this deadly bath of chemicals. It was thought that the stomach was sterile, but the discovery of *H. pylori* changed this idea. Acid resistance is crucial in the gastric niche, and more

than 300 genes are acid regulated or acid affected (McGowan et al., 2003). Also large amounts of a potent, multi-subunit urease enzyme are produced by *H. pylori*. This converts urea to ammonia and CO_2 leading to neutralisation of the surrounding environment (Melchers et al., 1998), the bacteria is surrounded by a layer of ammonia that neutralises hydrogen ions before lowering the intracellular pH to above acidic levels (Melchers et al., 1998). This allows *H. pylori* to survive in gastric acid so that it is able to colonise the gastric mucosa (Marshall et al., 1990).

H. pylori induces an infiltrate of T lymphocytes, plasma cells, mononuclear phagocytes, and neutrophils and stimulates the expression of proinflammatory cytokines, such as tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1 β), interferon gamma (IFNγ), IL-4 and IL-8 (Atherton, 2006, Bergman et al., 2004, Crabtree et al., 1995, Mohammadi et al., 1997, Sawai et al., 1999, Smythies et al., 2000, Yamamoto et al., 2004, Yamaoka et al., 2006). Despite a specific humoral and cellular immune response, the infection shows lifelong persistence in a majority of cases (Atherton, 2006). The inability to eliminate *H. pylori* may be due to bacterial virulence determinants and immune evasive strategies as well as an inappropriate host immune response. Gastric pathology appears closely associated with *H. pylori* virulence genes (Atherton, 2006, Kidd et al., 2001a, Kidd et al., 2001b, Qiao et al., 2003). The two most important virulence factors are encoded by the vacuolating cytotoxin gene (*vacA*) and the cytotoxin associated gene (*cag*) pathogenicity island (PAI) (Atherton, 2006, Atherton, 1998).

1.2 Epidemiology and Transmission

H. pylori colonises all human populations worldwide (Cover et al., 2001). The socioeconomic status, geographic area and age are important factors and play a crucial role in the risk of colonisation and disease development. Initial colonisation is thought to occur during early childhood, particularly in developing countries (Cover et al., 2001).

Although the infection is almost ubiquitous in Third world countries, in developed countries *H. pylori* infects 30–50% of adults (Atherton, 2006, Cover et al., 2001). The decrease in *H. pylori* infection rate associated with industrialization and improvements in socioeconomic levels is due to the improvements in sanitation, and reduced crowding. 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 risk of being infected (Atherton, 2006, Blaser et al., 2007, Cover et al., 2001).

No significant reservoirs of *H. pylori* exists outside the human stomach (Blaser and Atherton, 2004), since it has a rather small genome which does not support all metabolic pathways necessary for a nonparasitic life style (Alm et al., 1999, Blaser and Atherton, 2004, Cover et al., 2001, Tomb et al., 1997). Person to person contact involving ingestion of *H. pylori* from saliva, vomitus, faeces or recently contaminated foods would be the most likely mode of transmission (Blaser and Atherton, 2004, De Schryver et al., 2006, Delport and van der Merwe, 2007, Lehours and Yilmaz, 2007, Perry et al., 2006). Serological and DNA fingerprinting analyses have shown that person to person transmission occurs mainly within families i.e. vertical transmission instead of horizontal transmission (Drumm et al.,

1990, Nguyen et al., 2006). In support of this, the same strain is frequently shared between mothers and their children, but less frequently between spouses. Interestingly, transmission is less common between fathers and their children (Han et al., 2000, Kivi et al., 2003).

1.3 Infection and Gastric Diseases

1.3.1 Gastritis

Healthy stomach mucosa contains very few inflammatory cells. However, when *H. pylori* colonises the stomach, polymorphonuclear cells and neutrophils migrate to the area resulting in an acute inflammatory response (Atherton, 2006). Hypochlohydira, or reduced gastric acidity, results from the acute infection. If these initial responses fail to clear the infection, neutrophils, T cells, B cells and macrophages accumulate in the gastric mucosa (Brandt et al., 2005, Tham et al., 2001), which is a characteristic histological picture of chronic active gastritis (Atherton, 2006). Most strains of *H. pylori* persist lifelong if not eradicated with antibiotics and all cause gastric inflammation (Atherton, 2006). The vast majority of patients stay asymptomatic without further progression. However, 15% of infections result in peptic ulceration and 0.5%–2% in gastric adenocarcinoma (Atherton, 2006, Dixon, 2000, Everett et al., 2002, Goldstone et al., 1996, Graham, 1997, Hida et al., 1999). Three factors interact together to determine whether subjects develop such diseases; the virulence of the infecting *H. pylori* strain, the type and the extent of the host immune response to infection, and modulating cofactors such as smoking and diet (Atherton, 2006, Graham, 1997).

1.3.2 Gastric Ulcer

Gastric ulceration (GU) is associated with a pan-gastritic inflammation pattern and reduced or normal acid production (Atherton, 2006, Dixon, 2001, Dixon, 2000). GU develops most commonly at the transitional zone between antrum and body on the lesser gastric curve. *H. pylori* is found in 80% of patients with gastric ulcer (Atherton, 2006, Dixon, 2001, Dixon, 2000). In individuals with normal or high

acid secretion, *H. pylori* does not normally colonise the corpus because of the low pH. However, when there is low acid secretion, the colonisation will be consistently spread throughout gastric mucosa (Dixon, 2001, Dixon, 2000). Colonisation by *H. pylori* leads to progressive inflammatory cell infiltration, and rising exfoliation of the epithelial cells. These changes lead to ineffective mucin and bicarbonate production, which weakens the mucus barrier and the tissue becomes more susceptible to ulceration (Atherton, 2006, Dixon, 2001, Dixon, 2000).

1.3.3 Duodenal Ulcer

Duodenal ulcer (DU) is associated with dense *H. pylori* infection and severe inflammation but only when inflammation is largely confined to the antrum, probably as a result of increased basal acid output and a heightened parietal cell response to stimulation. *H. pylori* is found in 95% of patients with duodenal ulcers (Atherton, 2006, Dixon, 2001, Dixon, 2000, Fikret et al., 2001). Excessive acid secretion into the duodenal lumen promotes the migration of gastric mucosa into the duodenum a condition which is called gastric metaplasia (Fikret et al., 2001). The appearance of gastric epithelial cells in the duodenum allows colonisation by *H. pylori*, which will establish a chronic inflammatory response (Fikret et al., 2001). The inflammation process and bacterial effect on the epithelial cells render the duodenal mucosa sensitive to gastric acidity, and thus predisposes it to ulceration (Atherton, 2006, Dixon, 2001, Dixon, 2000, Fikret et al., 2001).

1.3.4 Gastric Cancer

Gastric adenocarcinoma is the second highest cause of cancer deaths worldwide (Atherton, 2006, GLOBOCAN, 2002). A possible explanation is the high prevalence of *H. pylori* infection (Correa, 2003, Correa et al., 1990, GLOBOCAN, 2002, Meining et al., 1998). Distal gastric adenocarcinoma (Correa, 2003, Correa et al., 1990, GLOBOCAN, 2002), which is *H. pylori* associated, is more common than the proximal form. In addition, *H. pylori* causes MALToma or B cell mucosa-associated lymphoid tissue (MALT) (Du and Atherton, 2006) lymphoma of the stomach (Bandipalliam, 2006, Iida et al., 2007, Moss and Malfertheiner, 2007). Gastric cancer arises as an outcome of long term gastric mucosal infection and chronic inflammation (Asaka et al., 1997, Blaser et al., 2007, Egi et al., 2007, Goldstone et al., 1996). Atrophic gastritis, intestinal metaplasia and dysplasia are preceding conditions of gastric cancer (Fikret et al., 2001). The difference in prevalence of infection *versus* incidence in gastric cancer (approx. 1%) suggests a multifactorial etiology such as differences in bacterial strains, host genotypes, and environmental conditions (Atherton, 2006, Peek and Blaser, 2002). In particular, strains that carry the *cag* PAI and virulent types of the vacuolating cytotoxin gene (*vacA*) are significantly associated with disease (reviewed in (Atherton, 2006)). Individual differences in host responses are also of major importance, and polymorphism in the pro-inflammatory cytokine interleukin IL-1β was the first described host risk factor for *H. pylori* associated gastric cancer (El-Omar et al., 2000, El-Omar et al., 2001). IL-1β is the most powerful inhibitor of gastric acid secretion known, and individuals who are colonised by *H. pylori* and possess “high expression” alleles of IL-1β run an increased risk of developing hypochlorhydria,

gastric atrophy and gastric adenocarcinoma (El-Omar et al., 2000, El-Omar et al., 2001).

On the other hand, there is some evidence against the association of *H. pylori* with gastric cancer. The strongest evidence comes from the unexplained African and Asian enigma where there is high *H. pylori* infection rate and low gastric cancer rate (Holcombe, 1992, Singh and Ghoshal, 2006). In a study conducted in two regions in China where there is high and low prevalence of gastric cancer, the prevalence of *H. pylori* infection was similar (Hu et al., 1995). Similarly, studies in some countries, Italy (Palli et al., 1993) and Costa Rica (Sierra et al., 1992), did not show a higher prevalence of infection among people living in regions with a high prevalence of gastric cancer.

Thus, both host and bacterial factors contribute to development of disease. Immune response to *H. pylori* also plays a major role. This will be discussed later. Furthermore, different environmental factors such as high salt intake and inadequate consumption of fruits and vegetables containing vitamin C has been regarded as risk a factor for development of gastric cancer (Asaka et al., 1997, Correa et al., 1998).

1.4 Histological Changes

1.4.1 Overview

Acute gastritis due to *H. pylori* is rarely seen in biopsy material because the infection is usually either asymptomatic or accompanied by minor gastrointestinal discomfort. The histological features include conspicuous pit abscesses and exudation of neutrophils into the surface epithelium (Owen, 2004). This is accompanied by marked epithelial degeneration and regeneration, which may be syncytial in type. The mucosa shows a dense infiltrate of chronic inflammatory cells, in which plasma cells are especially prominent. Lymphoid follicles with germinal centres are usually seen, particularly in the deeper portion of the mucosa. This finding is virtually pathognomonic for the presence of *H. pylori* (Owen, 2004). The surface and pit lining epithelium is infiltrated by neutrophils, which may be so prominent that pit abscesses are formed. This neutrophil infiltration is termed "active gastritis" and is seen predominantly in areas where the *Helicobacter* organisms are most abundant and most readily identified (Owen, 2004). The Modified Sydney system is used for the classification of gastritis (Misiewicz, 1991). This system introduces a semiquantitative grading system from 0 to 3, according to the severity, for *H. pylori*, neutrophil granulocyte, mononuclear cellular infiltration, atrophy and intestinal metaplasia. There are some limitations to this system such as difficulties of its usage in routine and every day reporting in a busy histopathology laboratory (Guarner et al., 1999). However, the system has been found useful and reproducible for treatment protocols and research purposes (Guarner et al., 1999).

In North America and Western Europe, most individuals with *H. pylori* infection have an active superficial gastritis, largely confined to the antrum, in which organisms are easily identified (diffuse antral gastritis or DAG) (Meining et al., 1998). This type of gastritis may result in duodenal ulcer formation, probably as a result of increased basal acid output and a heightened parietal cell response to stimulation (Dixon, 2000, Meining et al., 1998, Sotoudeh et al., 2008). It may also cause non ulcer dyspepsia, although this association is controversial (Atherton, 2006). In contrast, individuals in underdeveloped countries typically have an atrophic gastritis (multifocal atrophic gastritis), which is patchy in distribution and involves bot*H pylori*c and fundal mucosa (Meining et al., 1998, Sotoudeh et al., 2008). *H. pylori* organisms may be sparser in this form of gastritis, which is associated with gastric ulcer and gastric carcinoma (Egi et al., 2007, Meining et al., 1998, Sotoudeh et al., 2008). The reasons for this geographic difference are unclear. One possibility is that individuals in Third World countries acquire the infection at an earlier age: progression from non atrophic superficial gastritis to an atrophic gastritis simply relates to the duration of infection or the increased susceptibility of children's stomachs to sustain damage (Asaka et al., 1998, Atherton, 2006). Another plausible explanation is that additional, but as yet unspecified, dietary factors may be required to mediate damage leading to atrophy and intestinal metaplasia (Atherton, 2006).

Treatment of *H. pylori* gastritis with appropriate antibiotics will usually result in rapid elimination of organisms and abolition of the neutrophilic infiltrate. Chronic inflammation, however, may persist for several years before disappearing (Kubben et al., 2007, Malfertheiner et al., 2006, Romero-Gallo et al., 2008).

1.4.2 Intestinal Metaplasia

Intestinal metaplasia is defined as transformation of gastric epithelial cells into goblet cells, which normally line the surface of colonic tissue (Fikret et al., 2001). These changes appear most often as a consequence of continuous regeneration of tissue and abnormal stimulation of growth during chronic inflammation (Fikret et al., 2001). Both atrophic gastritis and intestinal metaplasia occur during long term (decades of) *H. pylori* infection and both are considered to be risk factors for development of cancer (Asaka et al., 1998, Fikret et al., 2001).

1.4.3 Atrophic Gastritis

Gradual loss of gastric glandular tissue as a consequence of long term mucosal destruction is called atrophic gastritis (Asaka et al., 1997, Atherton, 2006). The tissue damage may involve progressive loss of all specific mucosal cells including the acid producing parietal cells, pepsinogen producing chief cells and mucus producing gland cells (Asaka et al., 1997, Atherton, 2006, Sipponen, 1998, Tham et al., 2001). When these cell types have shrunk, the protective mucus layer will gradually disappear and acid secretion will cease (Atherton, 2006, Sipponen, 1998). Such pathological changes increase the risk of gastric ulceration and development of gastric adenocarcinoma (Fikret et al., 2001). However, this protects against duodenal ulcers because of low acid secretion (Atherton, 2006).

1.5 Virulence Factors

1.5.1 The *cag* PAI and *cagA* gene

The cytotoxin associated gene A (CagA) protein, which is encoded by the *cagA* gene, is a highly immunogenic protein. *H. pylori* strains possessing *cagA* are associated with a significantly increased risk for the development of atrophic gastritis, peptic ulcer diseases and gastric cancer (Atherton, 1998, Atherton, 1999, Atherton, 2000, Peek et al., 1997, Rokkas et al., 1999). The *cagA* gene is situated at one end of a 40 kb DNA insertion called the *cag* PAI and may have been acquired from a non-*Helicobacter* origin (Atherton, 2000, Censini et al., 1996). The *cag* PAI contains approximately 30 genes which are multicistronic. The difference in the ability of *H. pylori* strains to trigger chemokines from gastric mucosa depends upon the expression of genes within the *cag* PAI (Atherton, 2000, Censini et al., 1996, Tomb et al., 1997) .

Many of the genes in the PAI are thought to encode components of a bacterial type IV secretion system (T4SS) (Censini et al., 1996). Recent studies have shown that CagA is directly introduced into epithelial cells *via* the type IV secretion system, becomes phosphorylated on tyrosine residues by host cell Src family kinases and then stimulates cell signalling pathways (Censini et al., 1996, Stein et al., 2000). This, *in vitro*, leads to clear changes in cell shape resulting in what is called the "hummingbird" phenotype (elongated cell with long processes) (Bourzac et al., 2007, Kurashima et al., 2008, Ren et al., 2006, Segal et al., 1999, Tsutsumi et al., 2003). Such cytoskeletal changes might be induced *via* the activation of the hepatocyte growth factor receptor c-Met which is an oncogene (Churin et al., 2003). Furthermore, it was recently shown that phosphorylated CagA forms a

physical complex with SHP-2 phosphatase which is known to have a positive role in mitogenic signal transduction, and deregulates its enzymatic activity (Higashi et al., 2002b). Deregulation of SHP-2 by CagA may stimulate abnormal proliferation and movement of gastric epithelial cells through the activation of c-Fos and c-Jun (Meyer-ter-Vehn et al., 2000). In addition, the phosphorylation of CagA has a negative feedback loop by which the activated CagA inhibits Src kinase preventing unregulated signalling (Atherton, 2006, Stein et al., 2002). It is worth mentioning that un-phosphorylated CagA can disrupt the apical junctional complex between gastric epithelium cells possibly facilitating the acquisition of nutrients (Amieva et al., 2003).

According to the sequence constituting the SHP-2 binding site, CagA proteins can be sub-categorised into Western (C motif) and East Asian (D motif) types. The East Asian type CagA possesses stronger SHP-2 binding and transforming activities than the Western-type CagA (Azuma et al., 2004). CagA is translocated into epithelial cells where it becomes phosphorylated on tyrosine residues in EPIYA tyrosine phosphorylation motifs (TPMs). EPIYA motifs are frequently repeated within the variable region of the protein (Higashi et al., 2005, Naito et al., 2006). Strains possessing CagA with greater numbers of these repeats have been more closely associated with gastric carcinogenesis (Argent et al., 2004, Zhang et al., 2005). Phosphorylated CagA leads to epithelial cell elongation, which is dependent on the number of variable-region EPIYA motifs (Argent et al., 2004, Zhang et al., 2005). Thus, determination of the degree of CagA phosphorylation and the number of EPIYA motifs appears to be more important than detection of *cagA* alone (Argent et al., 2004, Argent et al., 2005, Zhang et al., 2005).

The C-X-C chemokine family, which comprises the polymorphonuclear cells (PMNL) chemo-attractant IL-8, is an important factor in the immunopathogenesis of gastritis (Crabtree et al., 1995, Hofman et al., 2000). High levels of gastric IL-8 are found in patients infected with *cagA* positive strains of *H. pylori* (Crabtree et al., 1995). Initial studies revealed that inactivation of 14 genes in the *cag* PAI reduced IL-8 secretion in epithelial cells (Crabtree et al., 1995), whereas the *cagA* gene seemed to be irrelevant (Crabtree et al., 1995, Sharma et al., 1998). However, a recent paper demonstrated that *cagA* could play a role in the induction of IL-8 secretion *via* a Ras-Raf-Mek-Erk-NF-*k*B signalling pathway in a SHP-2 and c-Met independent manner (Brandt et al., 2005). Another paper has shown that the T4SS facilitates the translocation of peptidoglycan into the host cell cytosol where it activates NOD1 which in turn activates the expression of IL-8 (Viala et al., 2004).

1.5.2 Vacuolating Cytotoxin

The vacuolating cytotoxin (VacA) is another *H. pylori* virulence factor (Atherton, 1998). VacA can induce apoptosis in epithelial cells, affect B lymphocyte antigen presentation, inhibit the activation and proliferation of T lymphocytes, and modulate the T cell-mediated cytokine response (Gebert et al., 2004). The toxin also induces the formation of large cytoplasmic vacuoles in epithelial cells (Cover, 1996). To a large extent, the function of this cytotoxin depends upon pH. At neutral pH, VacA congregates into large, water-soluble oligomeric complexes comprising 12 or 14 identical monomers (Cover et al., 1997). When exposed to acidic or alkaline pH, these oligomeric complexes disassemble into component monomers (Cover et al., 1997). Acid activated VacA can be integrated into lipid bilayers and

the plasma membrane of eukaryotic cells to form anion and urea selective membrane channels (Czajkowsky et al., 1999). This urea permease activity may help acid survival through supplying a substrate for urease (Atherton, 2006). Moreover, a recent study has demonstrated that VacA increased the permeability of the epithelial barrier, probably by acting at the tight junction level (Papini et al., 1998). However, VacA is not involved in *H. pylori* induced polymorphic nuclear lymphocytes (PMNL) migration across a tight epithelial barrier (Hofman et al., 2000).

Unlike *cagA,* almost all *H. pylori* strains posses the vacuolating cytotoxin gene (*vacA*) (Atherton, 2006). *vacA* is translated into a prepro-protein which is an autotransporter that undergoes N- and C-terminal cleavage during its secretion. The mature secreted VacA toxin comprises 2 subunits; p37 and p58, linked *via* a flexible loop region. It has a molecular mass of 88 kDa and consists of about 821 amino acids (Reyrat et al., 2000). Vacuole formation in HeLa cells can be induced by transfecting these cells with *vacA*-containing plasmids. Expression of the amino-terminal 422 amino acids, comprising the p37 domain and a part of the p58 domain, is sufficient to induce vacuole formation (de Bernard et al., 1998, Ye et al., 1999). Rabbit antiserum induced against a recombinant peptide containing amino acids 476 to 803 of VacA inhibits the binding of VacA to cells and neutralizes toxin activity (Ye et al., 1999). These data suggest that a carboxy-terminal VacA p58 domain performs binding of the toxin to cells and an amino-terminal domain mediates the intracellular activity of VacA (Nguyen et al., 2001, Ye et al., 1999).

The *vacA* gene contains at least two variable parts, the signal and middle regions. For the signal region, two sequence families, called s1 and s2, have been identified in different isolates. Among s1 strains, subtypes s1a, s1b, and s1c have been identified (van Doorn et al., 1998). Subtypes s1a and s1b differ in five amino acids (Ala18Val, Val20Ala, Val24Ile, Ile26Ala/Ser, and Thr27Ile). Subtype s1c differs from subtype s1a at two positions (Ala22Leu and Gln30Lys) and from s1b at seven positions (Ala18Val, Val20Ala, Ala22Leu, Ile24Val, Ala/Ser26Ile, Ile27Thr, and Glu/Gln30Lys) (van Doorn et al., 1998). Two alleles, m1 and m2, of the mid-region of the *vacA* gene have been described, and the m2 cytotoxin is inactive in the *in vitro* HeLa cell assay.

Vacuolating cytotoxin activity is related to the mosaic structure of *vacA*. In general, type s1/m1 and s1/m2 strains produce high and moderate levels of toxin activity, respectively, whereas s2/m2 strains produce no vaculating activity (Atherton et al., 1995). A 12-amino-acid hydrophilic amino-terminal segment, present in type s2 but absent from type s1 VacA proteins, slows the capacity of VacA to form membrane channels and abolishes vacuolation. Some type s1/m2 VacA toxins show cytotoxic activity toward selected cell types, including RK-13, but relatively little activity for HeLa or AGS cells (Atherton et al., 1995, Letley et al., 1999, van Doorn et al., 1998). Heterogeneity among *vacA* alleles may be an important factor in understanding variations in clinical manifestations among *H. pylori* -infected subjects. Several studies have demonstrated that gastric infection with *H. pylori* strains containing type s1 *vacA* alleles are associated with a higher risk for development of peptic ulcer disease than infection with strains containing type s2 *vacA* alleles (Atherton et al., 1995). This relation is not seen in Asia as an Asian

strains are vacA type s1 (Atherton et al., 1995, Han et al., 1998, Ito et al., 1997, Pan et al., 1998). Thus in these countries, s1 cannot be used as a marker for the presence of peptic ulcer disease because the prevalence of the s1 genotype is uniformly high.

Rhead and colleagues have described a novel determinant of VacA toxicity, called the intermediate or i-region (Joanne et al., 2007). They showed that two allelic variants of this region existed, i1, and i2. Furthermore, only s1/m2 strains varied in i-type; s1/m1 and s2/m2 strains were exclusively i1 and i2 respectively. This novel region determines vacuolating activity among these s1/m2 strains. More importantly, a significant correlation has been established between the i1 region and gastric cancer (Joanne et al., 2007). In contrast to Rhead *et al*., no disease association between *vacA* i genotypes and outcome was found in East Asian and Southeast Asian countries (Ogiwara et al., 2008). More studies, from other countries, are needed to determine whether this region is a true virulence determinant.

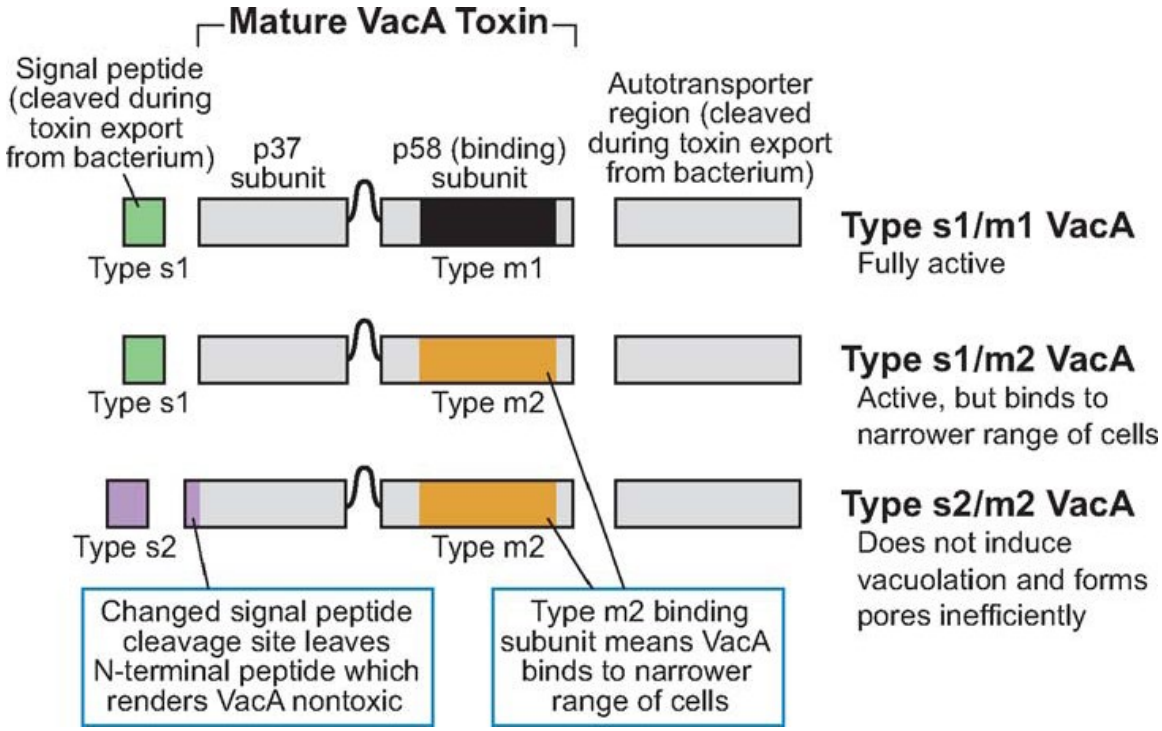

Figure 1. 1 Polymorphisms in VacA.

The *vacA* gene varies most markedly in the signal region (encoding the signal peptide), which may be type s1 or s2, and in the mid-region (encoding part of the p58 binding subunit). This may be type m1 or m2. Three types of toxin are commonly found: s1/m1, s1/m2, and s2/m2 (the s2/m1 combination occurs but is rare). (Atherton 2006).

1.5.3 Blood Group Antigen Binding Adhesin A

The BabA adhesin of *H. pylori* is an outer membrane protein that attaches to the fucosylated Lewis b histo-blood group antigen on the surface of gastric epithelial cells (Boren et al., 1993, Ilver, 1998). Within the gastric mucosa, *H. pylori* lies within the mucus layer and may also bind to gastric epithelial cells. Adherence to the epithelium is assumed to help protect the bacteria from gastric acidity, as well as from displacement due to peristalsis (Rad et al., 2002). Lewis b-dependent attachment of *H. pylori* to gastric epithelial cells is associated with increased severity of inflammation, development of parietal cell autoantibodies, and parietal cell loss (Rad et al., 2002). Two genes encoding BabA have been cloned: *babA1* and *babA2*, but only the *babA2* gene is functionally active. Only about half of the *H. pylori* strains tested produced a detectable BabA protein (Bjornham et al., 2005, Hennig et al., 2004, Pride et al., 2001). Among *H. pylori* strains that produce a detectable BabA protein, there is considerable variation in binding to Lewis b (Bjornham et al., 2005). In a study conducted in Brazil, a strong relationship between *babA2* and duodenal ulcer or gastric carcinoma was detected, even after adjusting for confounding factors, such as age, gender, and *cagA* status (Kim et al., 2001, Oliveira et al., 2003). Studies in Western countries have also revealed a strong relationship between *babA2*-positive status and duodenal ulcer and gastric carcinoma (Gerhard, 1999). However, in Asian countries, most of the circulating *H. pylori* strains are *babA2* positive, whether or not they were isolated from asymptomatic or diseased patients (Kim et al., 2001, Lai et al., 2002, Mizushima et al., 2001). In addition to these differences between Western and Eastern countries, the prevalence of *babA2*-positive *H. pylori* strains varies among Western populations, being much lower in subjects from Portugal (Oleastro et al., 2003)

than in those from Germany (Gerhard, 1999), or the United States (Backstrom et al., 2004). Triple positive phenotype strains (*babA2*, *cagA*, and *vacAs1*) are associated with a high prevalence of ulcer and adenocarcinoma (Gerhard, 1999, Hennig et al., 2004, Kim et al., 2001, Oliveira et al., 2003).

1.5.4 Outer Inflammatory Protein A

The *H. pylori* outer inflammatory protein, OipA, is a virulence factor associated with increased IL-8 secretion and augmented inflammation *in vitro* as well as the clinically important presentation of peptic ulcer (Yamaoka et al., 2000, Yamaoka et al., 2006) although some have failed to show this association (Akanuma et al., 2002). The gene *oipA* is found in all strains, although, variable numbers of dinucleotide repeats in its 5´ region mean that an active protein may or may not be produced; this adaptation system is called phase variation and is based on the slipped-strand repair mechanism. Switch "on" status is functional and "off" status is non-functional. Such status may affect bacterial characteristics such as virulence. Strains possessing the *cag* pathogenicity island typically also have OipA with functional status "on" (Kudo et al., 2004). PCR-based sequencing of the signal region of the gene is sometimes used to imply the presence of a functional OipA protein (Kudo et al., 2004). However, sequencing of the 5´ region of the gene does not guarantee that no mutations are present downstream that would prevent production of the protein, so detection of OipA expression by western blot analysis should also be performed.

1.5.5 Duodenal Ulcer Promoting Gene A (*dupA*)

Recently, duodenal ulcer promoting gene A, *dupA*, has been identified and shown to play a role in provoking IL-8 secretion (Lu et al., 2005a). The *dupA* gene is located in a region of the bacterial genome that encodes surface proteins and it is a VirB4 homologue (Lu et al., 2005a). Lu *et al.* showed that the presence of *dupA* was significantly associated with duodenal ulceration, but negatively associated with gastric cancer, in populations from South Korea, Japan, and Colombia. They also showed that it increased IL-8 secretion (Lu et al., 2005a). A population from northern India also demonstrated a significant association between the presence of *dupA* and duodenal ulceration, although no patients with gastric cancer were included in the study (Arachchi et al., 2007). In contrast, in a population from Brazil, no significant association between *dupA* prevalence and ulceration or cancer was found (Gomes et al., 2007). It was recently found, however, that *dupA* was not significantly associated with duodenal ulceration in populations from Belgium, South Africa, China, and the USA, but was significantly associated with gastric cancer development (Argent et al., 2007). This association with duodenal ulceration in some populations and gastric cancer in others is similar to the association of the *cag* PaI with disease. The *cag* PaI is thought to induce increased gastric inflammation largely through increasing IL-8 secretion from epithelial cells (Crabtree et al., 1995). Finally, an association between *dupA*-negative strains of *H. pylori* and pre-malignant lesions was found in Iran (Douraghi et al., 2008). The variation in the results calls for additional studies of *dupA* to evaluate the role of this gene in disease processes.

1.6 Genomic Diversity

H. pylori has a wide genetic diversity amongst isolates from different subjects. It is extremely difficult to find two isolates, from unrelated individual, with identical genomic fingerprints by methods such as random amplification of polymorphic DNA (RAPD) (Akopyanz et al., 1992). Additionally, genomic comparison of different strains has shown that 7% of the genes are specific for each strain (Alm et al., 1999). Such a diversity is conferred by many mechanisms, including 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, Falush, 2001, Suerbaum and Josenhans, 2007). The recombination rate in *H. pylori* is the highest among all bacteria that have been studied so far (Suerbaum and Josenhans, 2007).

H. pylori is naturally competent for transformation, which allows uptake of free DNA released from nearby strains in the local environment when they die. Foreign DNA fragments undergo recombination for integration into the chromosome. *comB* is believed to play an important role in the process of natural transformation (Hofreuter et al., 2001). Uptake of foreign DNA and subsequent homologous recombination results in genetic diversity. Interestingly, the average size of recombined fragments in *H. pylori* (about 400 bp) is much smaller than in other bacteria (Falush, 2001).

The 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 most host adapted bacterial species.

According to their geographical distinction, *H. pylori* can be divided into four populations; hpAfrica 1, which is subclassified into hspWAfrica and hspSAfrica, hpAfrica 2, hpEastAsia, which is subdivided into hspAmernid, hspEAsia and hspMaori, and hpEurope. These modern populations derive their gene pools from five ancestral populations; Africa1, Africa2, EastAsia, Europe1 (AE1), and Europe2 (AE2). Human migration throughout history played the major role in subsequent spread (Falush et al., 2003).

The *vacA* s1a or s1b genotypes are predominant in strains from Western countries, whereas s1c is highly prevalent in strains from East Asia (van Doorn et al., 1999). The *vacA* m1a and m2a genotypes are predominant in strains from Western countries, whereas the m1c genotype is predominant in strains from South Asia, and the m1b and m2b genotypes are predominant in strains from East Asia (van Doorn et al., 1999). Overall, strains from Western countries predominantly possess *cagA*, *vacA* s1a, s1b, or s2 and m1a or m2a genotypes (van Doorn et al., 1999). Strains from South Asia predominantly possess *cagA* and *vacA* s1a/m1c genotypes, whereas strains from East Asia predominantly possessed *cagA*, *vacA* s1c/m1b, or m2b genotypes (van Doorn et al., 1999). These variations in the global distribution of the *cagA* and *vacA* genotypes may explain the diversity of reports associating the *cagA* and *vacA* genotypes with the clinical outcome from different geographic regions (Atherton, 2006). Other preliminary studies have supported the finding that *vacA* s2 strains are rarely associated with ulcers but have also found that such

strains are uncommon in many populations (Atherton, 2006, Atherton et al., 1997). More than 80% of Africans are infected with *H. pylori*, (Holcombe, 1992). In Ethiopia, for example, the *vacA* gene was detected in all *H. pylori* strains and the *vacA* genotype s1/m1 was found in 48% of the strains and 79% were *cagA* positive (Asrat et al., 2004). In spite of this, gastric cancer rates are lower than might be predicted based on this high prevalence which may indicating the presence of an enigma in this region (Holcombe, 1992).

1.7 Immune Response

1.7.1 T Helper cells 1 (Th1) and 2 (Th2)

The Th-0 naïve cells may be differentiated into certain Th subsets (Th1, Th2, Th17, Treg) depending on the cytokine environment where antigen presentation occurs. Th cells are an important regulator of the immune system, for example they control inflammation and B-cell memory. Usually Th2 cells are responsible for dealing with extracellular bacterial and parasite infections, whereas intracellular infections, including viruses, are dealt with by Th1 cells. Th1 cells, whose maturation is stimulated by interleukin-12 (IL-12), predominantly secrete interferon gamma (IFNγ) and IL-2, whilst Th2 cells, which may be stimulated by IL-10, secrete IL-4, IL-5, IL-9, and IL-13 (Atherton, 2006, Bergman et al., 2004, Robinson et al., 2008, Smythies et al., 2000). Th1 and Th2 cells cross-regulate one another. IFNγ secreted by Th1 cells directly suppresses IL-4 production, and thus inhibits the differentiation of naïve Th cells into Th2 cells (Gajewski and Fitch, 1988). In contrast, IL-4 inhibits the secretion of IFNγ and blocks the activity of Th1 cells (Ohmori and Hamilton, 1997).

In *H. pylori* infection, although both Th1 and Th2 arms of the immune response are provoked, gastric mucosal cytokine profiles suggest that the Th1 response predominates (Andersen and Wadström, 2001, Atherton, 1998, Whitfield, 2003) Th1 cells are induced by the actions of IL-12 during antigen presentation to the naive T-cell. *H. pylori* neutrophil-activating protein (HP-NAP) was shown to play an important role in stimulating IL-12 production from neutrophils and monocytes, biasing the immune response towards Th1 (Amedei et al., 2006). Studies in animal models and supportive data in humans show that a strong Th1 response increases

the severity of gastritis and cancer risk (Akhiani et al., 2002, Bamford et al., 1998, Blaser, 1992). In *IFNγ* knock-out mice a lesser inflammatory response to *H. pylori* was found in comparison to wild-type mice, indicating the importance of IFNγ for exacerbating gastritis (Akhiani et al., 2002). With *H. pylori* induced Th2 responses there is milder gastritis; a higher *H. pylori* colonisation density and lower risk of gastric cancer (Atherton, 2006) (Figure 1.1).

H. pylori induces a strong T-cell mediated immune response yet it is ineffective in clearance. Atherton argued that such an intense immune response is deleterious to the bacteria and it might cause elimination of infecting micro-organisms in mice (Atherton, 2006, Kotloff, 1996). However in humans *H. pylori* appears to downregulate the Th responses to avoid clearance (Atherton, 2006). This balance between the up and the downregulation of Th1 may be achieved via several mechanisms. Firstly, it was found that Lewis antigen can bind to the dendritic cell specific intracellular adhesion molecule-3 (ICAM3) binding nonintegrin (DC-SIGN) lectin on gastric dendritic cells; this binding halts Th1 cell development. However, not all *H. pylori* carry Lewis antigens, therefore, it is expected that Lewis negative variants induce stronger Th1 response than Lewis positive variants (Atherton, 2006, Bergman et al., 2004, Rad et al., 2002) . Secondly, Th1 polarisation can be diminished through *H. pylori* stimulated cyclooxygenase-2 production by mononuclear cells (Byrne et al., 2003, Meyer et al., 2003, Wight et al., 2001). Thirdly, *H. pylori* can stimulate a regulatory T cells (Treg) response (Lundgren A et al., 2003). In the presence of the Treg cells, lower levels of IFNγ are secreted by memory Th cells in *H. pylori* infected subjects than those from *H. pylori* uninfected people (Atherton, 2006, Lundgren A et al., 2003). Finally,

external factors can play a role in downregulating the Th1 response. It has been shown that the coinfection of mice with *Helicobacter felis*, a *Helicobacter* species which has wider host range than *H. pylori* (Lee et al., 1990, Lee et al., 1988, Lockard and Boler, 1970), and helminths, leads to increased *H. felis* density, but reduced Th1 cytokine levels (Fox JG et al., 2000). On the other hand, the immune response in mice co-infected with *H. felis* and *Toxoplasma gondii* was pushed towards a Th1 biased response. Gastric mucosal IFNγ and IL-12 levels were significantly elevated and IL-10 expression was substantially reduced (Stoicov et al., 2004).

There are reported variations in the immune response to *H. pylori* infection in different human populations. The immune response to *H. pylori* infection in an African population was predominantly Th2 and differed from that observed in subjects from Europe which was mainly Th1 (Mitchell et al., 2002). Other researchers have shown evidence of a Th2 response especially in children (Campbell et al., 2004). In addition, previous studies have shown that the Th2 response is stronger in women (Ansar et al., 1985).

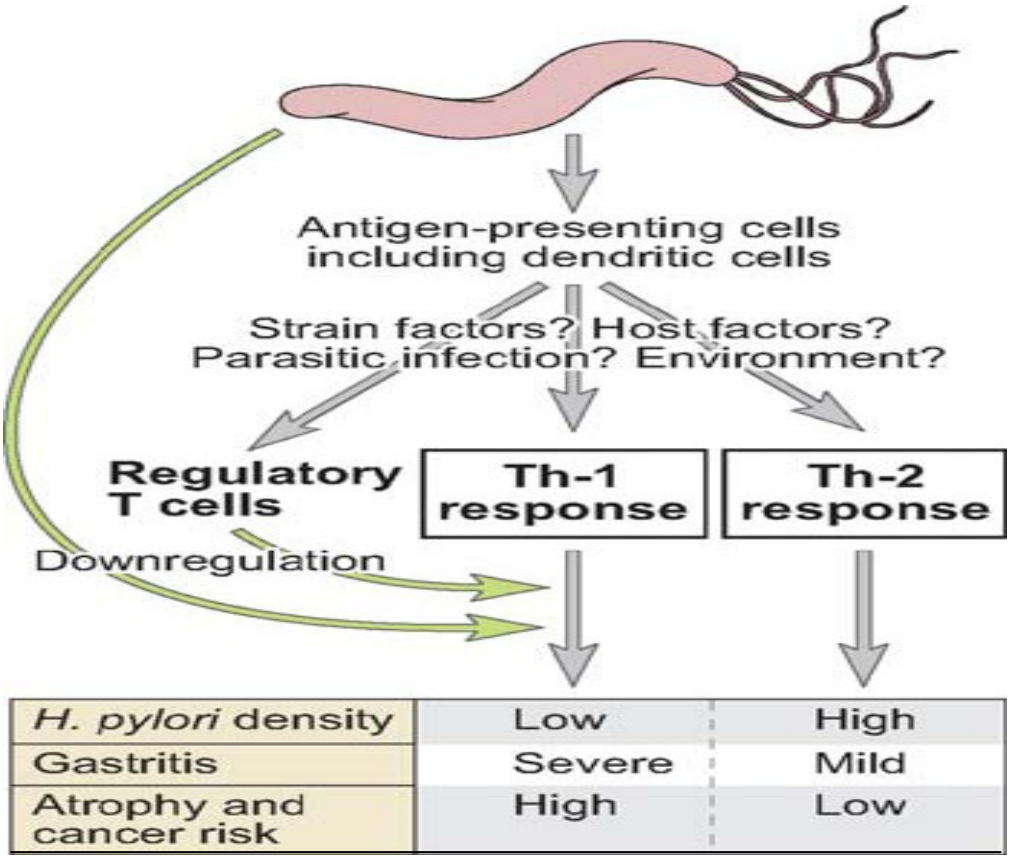

Figure 1. 2 The central role of the Th cell response to *H. pylori* infection.

Th1, Th2, and regulatory T cells are activated, but the balance of the response varies between people owing to unknown factors. Studies in animal models and supportive data in humans show that a strong Th1 response increases severity of gastritis and cancer risk. Both *H. pylori* and the host downregulate this Th1 response. This potentially benefits *H. pylori* by allowing more dense colonization and benefits the host by downregulating mucosal inflammation. (Atherton 2006).

1.7.2 Regulatory T cells (Treg)

The immune system can distinguish between self and non-self antigens, preserving self-tolerance and preventing a damaging immune attack on the body's cells and tissues. This is achieved via several mechanisms. Firstly, potentially dangerous self-reactive T and B cells are clonally removed at primitive stages of their development or inactivated when encountering self-antigens. Secondly, a population of self-reactive CD4+ T cells, called regulatory T cells (Tregs), participate in the maintenance of peripheral self-tolerance by blocking the activation and expansion of damaging self-reactive T cells. Treg cells constitute 5–10% of CD4+ T cells and the vast majority of such naturally occurring CD4+ cells constitutively express high levels of CD25 (Hori et al., 2003, Sakaguchi, 2000, Sakaguchi et al., 1995, Sakaguchi et al., 2001).

It has been shown that Treg cells are able to suppress the proliferation and cytokine production of both CD4 and CD8 T cells via a cell-cell contact-dependent mechanism and/or the secretion of anti-inflammatory cytokines such as IL-10 and transforming growth factor β (TGFβ) (Sakaguchi et al., 2001). Additionally, the *in vitro* suppressive activity of Treg cells can be induced by several different types of antigens, including dietary, self, and foreign antigens (Taams et al., 2002). Furthermore, Treg cells have been demonstrated to be responsible for maintaining tolerance to food antigens (Lundin et al., 1999, Zhang et al., 2001) and to the normal intestinal flora (Singh et al., 2001). Experiments on mice have shown that the removal of this population of cells leads to spontaneous development of various autoimmune diseases in otherwise normal mice (Sakaguchi et al., 1995). This uncontrolled immune reaction has been noticed in humans as well. the best

example is IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome) (Gambineri et al., 2003). The *FOXP3* gene encodes a transcriptional repressor and acts as the master regulator in the development and function of Treg cells (Bennett et al., 2001, Brunkow et al., 2001, Chatila et al., 2000, Fontenot et al., 2003, Hori et al., 2003, Khattri et al., 2003, Wildin et al., 2001). Furthermore, transfusion of $CD25^{+}CD4^{+}$ T cells prepared from normal mice can prevent autoimmune disease in *FOXP3*-defective mice (Fontenot et al., 2003). Walker and colleagues argued that the discovery of *FOXP3/FOXP3* as a marker for natural $CD25^{+}CD4^{+}$ cells made it possible to determine the origin and the developmental pathway of these cells (Walker et al., 2003). Unfortunately, FOXP3 expression can be induced in non-Treg cells so it is not a completely specific and stable marker, however it is widely regarded as the best marker available at this time (Fontenot et al., 2003, Hori et al., 2003).

Treg responses increase with age. Two theories explain the origin of $CD25^{+}CD4^{+}$ cells. One is that naive T cells can differentiate to $CD25^{+}CD4^{+}$ cells upon T-cells receptor stimulation, in a manner similar to that of Th1 and Th2 cells (Apostolou et al., 2002, Thorstenson and Khoruts, 2001). Another theory is that some of the functionally mature regulatory T cells produced by the thymus are $CD25^{-}$ or lose CD25 expression with retention of their suppressive function. In rodents, such $CD25^{-}$ cells become $CD25^{+}$ when activated (Annacker et al., 2000, Gavin et al., 2002) (Stephens and Mason, 2000).

It was recently shown that Treg cells reduce *H. pylori*-induced gastritis in mice and humans (Couper et al., 2008, Grencis et al., 1991, Raghavan et al., 2003). These

cells can also halt the induction of colon cancer in mouse models by inhibiting the inflammatory response to *H. hepaticus* (Erdman et al., 2003). The induction of Treg cells may halt the process of disease progression by suppression of the damaging immune and inflammatory response. On the other hand, this suppression may allow bacterial persistence and the effectiveness of immunosurveillance against tumour cells may be diminished. In support of this, a relationship between Treg cells and the progression of tumours was shown (Karanikas et al., 2008, Karube et al., 2004, Miller et al., 2006, Rao et al., 2006) and high levels of these cells are regarded as a poor prognostic sign in cancer patients (Liu et al., 2008). Furthermore, Treg activity is a marker of tumour progression and metastasis in breast carcinoma (Gupta et al., 2007).

1.7.3 T helper cells-17 (Th17)

Recently, a novel pathway of inflammation characterized by excessive production of IL-17 has been described in a variety of chronic inflammatory diseases such as multiple sclerosis (Weaver et al., 2007). Th17 cells differentiate and grow in the presence of a combination of TGFβ1 and IL-6, together with IL-23 secreted from antigen presenting cells (Caruso et al., 2007, Di Sabatino et al., 2008). These CD4+ cells have been designated Th17 based on their production of IL-17 which is not produced by Th1 or Th2 cells. In addition, Th17 cells produce a range of other factors known to drive inflammatory responses, including tumor necrosis factor-α (TNFα), IL-6, and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Kolls and Linden, 2004, Park et al., 2005, Steinman, 2007, Weaver et al., 2007). In a study conducted using human cells, it was shown that Th17 cells share major trafficking receptors with both polarized effector T cells and FOXP3+ regulatory T

cells (Lim et al., 2008). The key contribution of the Th17 cell subset to immune responses against *H. pylori* remains undefined (Caruso et al., 2007). However, Kullberg *et al.* recently showed, in two models of *H. hepaticus* triggered T cell-dependent colitis, that IL-23 drives both IFNγ and IL-17 responses that together synergize to trigger severe intestinal inflammation (Kullberg et al., 2006). In another experiment, it was shown that IL-23 is over-expressed in *H. pylori*-infected gastric mucosa where it could contribute to sustaining IL-17 production (Caruso et al., 2008). In addition, it was shown that IL-23 enhances IFNγ synthesis in normal gastric lamina propria mononuclear cell (LPMC), and that anti-IL-23/p19 diminishes the production of IFNγ in LPMC of *H. pylori*-infected patients (Caruso et al., 2008). More studies are needed to explore the role of Th17 in the disease development process in *H. pylori* infected patients.

1.7.4 Interferon gamma and Interleukin-4

Interferon gamma (IFNγ) is a naturally occurring protein produced by Th1 cells to regulate and boost the immune system *via* its cellular axis (Atherton, 2006). *H. pylori* infection stimulates Th1 cells to produce IFNγ which in turn plays an important role in the disease process (Sommer et al., 2001, Yamamoto et al., 2004). *H. pylori* infected mice with defects in IFNγ expression (IFNγ null mice) develop less severe gastric inflammation and have higher bacterial colonization densities (Sommer et al., 2001, Yamamoto et al., 2004). The infusion of IFNγ alone can induce gastric inflammation and atrophy. However, the influence of this cytokine is enhanced with *Helicobacter* co-infection (Cui G, 2003). This implies that IFNγ plays a role in increased severity of *H. pylori*-induced gastric inflammation and also reduces bacterial colonization. In addition, IFNγ modulates the severity of

gastric inflammation indirectly via macrophage activation and also may down-regulate the expression of anti-inflammatory factors such as transforming growth factor β (TGFβ) (Strober et al., 1997, Yamamoto et al., 2004).

Interleukin-4 (IL-4) is a protein produced by activated Th2 cells to promote antibody production. *H. pylori*-infected IL-4-/- mice developed more severe gastric inflammation than did *H. pylori*-infected wild-type mice implying that the Th balance was tipped towards Th1 in the absence of IL-4 leading to increased inflammation (Smythies et al., 2000). Similarly, *H. felis*-infected IL-4 null mice developed significantly more severe gastritis than did *H. felis*-infected wild-type mice (Mohammadi et al., 1997). These data suggested that IL-4 plays a role in down-regulating gastric inflammation.

Gastrin, which is a hormone that stimulates gastric acid secretion, is stimulated by IFNγ. In contrast, the expression of somatostatin, a hormone that inhibits gastric acid secretion, is activated by IL-4 and inhibited by IFNγ and TNFα (Beales et al., 1997, Zavros et al., 2003). Hence, a Th1 response is expected to exacerbate gastrin production, whereas expression of IL-4 (a Th2 response) is expected to result in increased somatostatin production and reduced gastrin secretion.

1.7.5 Interleukin-8

Interleukin-8 (IL-8) is a powerful chemotactic factor for neutrophils and lymphocytes. This chemokine is secreted by antigen presenting cells and endothelial cells (Utgaard et al., 1998, Wolff et al., 1998). IL-8 promotes the adhesion of neutrophils to vascular endothelial cells. Then neutrophils migrate and

enter the infected and inflamed tissue. When *H. pylori* comes into contact with epithelial cells, these cells start to produce cytokines such as IL-8 (Nozawa et al., 2002). The *cag* PaI is known to induce high levels of IL-8 secretion from the gastric epithelium (Censini et al., 1996, Crabtree et al., 1995, Keates et al., 1997). OipA has also been shown to contribute to IL-8 secretion (Yamaoka et al., 2002a, Yamaoka et al., 2002b, Yamaoka et al., 2000, Yamaoka et al., 2006), although some have failed to show this association (Akanuma et al., 2002). Recently, *dupA* has been suggested to be an IL-8 inducer (Lu et al., 2005a). Polymorphisms that increase expression of the *IL-8* gene have been found to be associated with increased risk of duodenal ulcer and nasopharyngeal carcinoma (Ben Nasr et al., 2007, Gyulai et al., 2004).

1.7.6 Interleukin-12 (IL-12)

Interleukin-12 (IL-12) is a heterodimeric cytokine produced by antigen presenting cells in response to intracellular pathogen infection. It helps in the differentiation of naïve T cells into Th1 cells, triggering the production of IFNγ. Together IFNγ and IL-12 not only induce the differentiation of Thl cells but also inhibit Th2 responses (Kubin et al., 1994, Ozmen et al., 1994, Trinchieri, 2003). In addition, vaccine-induced protective immunity is IL-12 dependent (Akhiani et al., 2002). IL-12 is formed from two subunits: the p35 (also found in IL-6) and p40 (also found in IL-23) subunits are encoded by *IL-12*A and *IL-12*B genes. The production of both subunits is required to form a biologically active heterodimer (p70) (D'Andrea et al., 1992, Harrington et al., 2006, Trinchieri, 2003). In addition, an association was found between *IL-12* polymorphisms leading to increased expression and gastric cancer risk, but not with intestinal metaplasia (Navaglia et al., 2005).

Dendritic cells are a group of specialised antigen-presenting leukocytes that perform essential functions in the development of immune responses. These cells are found in the skin, lymphoid tissue and mucosa including the gastrointestinal mucosa. They represent the first line of the immune system that interacts with *H. pylori*, a reaction that elicits IL-12 secretion (Cutler et al., 2001, Wick, 2002, Yrlid and Wick, 2002). IL-12 upregulation in the gastric mucosa is associated with *cag* PAI-positive strains and leads to a Th1-biased response (Hida et al., 1999). A Th1 oriented response has been implicated in perpetuating the inflammatory changes that lead to disease (Bergman et al., 2004, Smythies et al., 2000, Trinchieri, 2003).

1.7.7 Interleukin-10

Interleukin-10 (IL-10), formerly known as cytokine synthesis inhibitory factor, inhibits the synthesis of IFNγ, IL-2 (which stimulates the differentiation of naïve T cells into Th1), IL-3 (which supports growth and differentiation of T cells from the bone marrow) and TNFα (which is involved in systemic inflammation and is a member of a group of cytokines that all stimulate the acute phase reaction). IL-10 has also been shown to have angiogenic activities (Keates et al., 2007). It is mainly secreted by Th2, monocytes and mast cells. However, it was recently shown that Th17, Treg and Th1 cells also can secret IL-10 (Couper et al., 2008, Steinman, 2007). Additionally, IL-10 can downregulate the expression of the major histocompatibility complex molecules (Bogdan et al., 1991, D'Andrea et al., 1993, de Waal Malefyt et al., 1991a, de Waal Malefyt et al., 1991b, Fiorentino et al., 1991), thus interfering with antigen presentation.

Antibodies blocking IL-10 production enhance the production of IFNγ by PBMCs in response to microbial antigens (Carvalho et al., 1994, Flores Villanueva et al., 1994). Additionally, *H. felis*-infected IL-10 knock-out mice develop a very severe gastritis (Berg et al., 1998). Furthermore, The inflammatory response in mice deficient in IL-10 is enough to clear *H. pylori* successfully and rapidly (Chen Wangxue et al., 2001). Hence, IL-10 may promote the colonisation of *H. pylori*. Finally, *IL-10* polymorphisms which cause lower than normal expression levels were associated with an increased risk of intestinal metaplasia (Zambon et al., 2005).

1.7.8 The Antibody Response to *H. pylori* Infection

Antibodies are protein (immunoglobulin) molecules produced by plasma cells and are responsible for humoral immunity. Antibodies are made from different classes of immunoglobulins. In mammals there are five antibody classes known as IgA, IgD, IgE, IgG and IgM. *H. pylori* provokes the induction of specific antibody response that consists mainly of IgA and IgG (Bontkes et al., 1992). There is some argument in the literature about the role of IgA in the diagnosis of *H. pylori*. While Bhat *et al.* showed that the mucosal CagA-specific IgA antibodies are produced during the acute phase of gastric inflammation (Bhat et al., 2005, Kullavanijaya et al., 2004) and are of poor sensitivity, Matsukura *et al.* showed that the tissue *H. pylori* IgA antibody assay is useful for detection of local immunity against *H. pylori* in the stomach and during follow-up after treatment (Matsukura et al., 1995). Nearly all infected individuals (>90%) exhibit *H. pylori*-specific IgG antibodies (Blaser, 1992) and these can be used for diagnosis of infection (Lenzi et al., 2006). It was shown that the main human IgG subclasses antibodies associated with *H.*

pylori infection are IgG2 and IgG1 (Bontkes et al., 1992) which are considered markers of a Thl and Th2 immune response, respectively (Mitchell et al., 2002, Vorobjova et al., 2006). The association between IgG subclasses and Th subset is stronger in mice than humans (Mosmann and Coffman, 1989, Yan et al., 2000).

1.7.9 Sex Hormones and the Immune System

As sex hormones differ between genders, many researchers have studied the effect of sex hormones on the immune response. It has been shown that women produce a stronger Th2 cellular and humoral immune response and suffer from a higher incidence of Th2-associated autoimmune diseases e.g. multiple sclerosis (Ansar et al., 1985). Faas *et al.* argued that the production of IL-4 is significantly higher in the luteal phase of the menstrual cycle as compared with the follicular phase (Faas et al., 2000). In another study, no differences in the percentage of lymphocyte subtypes during the menstrual cycle could be found (Bouman et al., 2005). However, post-menopausal women showed a reduction of the number of total lymphocytes in comparison to fertile women implying a role for progesterone and oestrogen (Giglio et al., 1994, Yang et al., 2000). In addition, T cell cytokine profiles could be modulated by pregnancy hormones: a high dose of progesterone present during pregnancy induces the production of IL-4 and biases the immune response towards a Th2 response (Zenclussen, 2006). Recently it was shown that there is an increase in the number of Tregs during pregnancy and failure in this augmentation could result in immunological rejection of the foetus. This could be prevented by adoptively transferring CD4+/CD25+ Treg cells from normal pregnant mice into abortion-prone animals (Zenclussen, 2005).

Bouman *et al.* concluded that there are no major effects of sex hormones on lymphocyte IFNγ secretion, and no differences in the production of IFNγ by male lymphocytes as compared to female lymphocytes. There was also no variation in the production of IFNγ during different phases of the menstrual cycle (Bouman et al., 2005). On the other hand, it is agreed that T lymphocyte counts are lower in males as compared to females which, perhaps is due to the increased testosterone concentrations, as this is associated with apoptosis in T cells (McMurray et al., 2001). In another study it was shown that oestrogen regulates the expression of IFNγ (Fox et al., 1991).

To summarise, differences in hormone profiles might affect the immune responses, changing their behaviour towards microorganisms and might skew the immune response in a manner resulting in a better or worse prognosis.

1.7.10 Smoking and Immune System

It was shown that smokers are more prone to peptic ulceration and gastric cancer than non-smokers (Ko and Cho, 2000, Maity et al., 2003, Sonnenberg, 1988, Walker and Taylor, 1979). Probably, smoking is associated with those diseases because it modulates the immune system. In an *in vitro* study it was shown that tobacco exposed lymphocytes have reduced proliferative capacities, which might affect the ability of the production of protective immunoglobulins against oral pathogens (Barbour et al., 1997). In addition, smoking is considered as the most powerful independent risk factor for invasive pneumococcal disease amongst immunocompetent subjects suggesting that smoking might modulate defence mechanisms of the lung (Nuorti et al., 2000). In a case control study, the effect of

smoking on natural killer cells, serum and saliva immunoglobulin level was studied. It was shown that there was a significant increase in NK cells activity against cultured melanoma cells in subjects who ceased smoking. Serum IgG and IgM levels increased significantly in those who gave up smoking, but not IgA. In saliva, the level of IgA and IgG rose significantly in subjects who stopped smoking. These changes could not be observed in those who continued to smoke. In this study, the mechanism behind those changes was not studied (Hersey et al., 1983). Furthermore, in a study conducted using lung biopsies from patients with obstructive airway diseases, it was shown that the activation of the transcription factor signal transducer and activator of transcription (STAT)-4, which is critical for the differentiation of Th1 and the production of IFNγ, is associated with smoking (Di Stefano et al., 2004). The importance of this research comes from the fact that IFNγ is associates with gastric metaplasia and atrophy both of which are premalignant conditions. In another study, smoking was proven to lower CD8 cell counts in smokers (Fatima et al., 1991). Furthermore, epidemiological associations have been shown between smoking and gastric cancer. However, it is not clear why smoking might increase the risk of cancer.

These findings suggest that there is an association of smoking with an increased incidence of certain malignant diseases probably through modulating the immune system.

1.8 Rationale and Aims

The gastric cancer rate in Iraq and especially in Kurdistan region is very low (GLOBOCAN, 2002) (Figure 1.3). In support of this, examination of the medical records for the period 2005-2006 at two general hospitals, Sulaimanyia hospital and Azadi hospital (hospitals servicing 2 million people), showed that only approximately 40 gastric cancers were diagnosed during that period in both hospitals. No studies have been conducted in this. The most obvious explanation could be low *H. pylori* prevalence, and this was investigated first. In addition, *H. pylori* infections are usually acquired in childhood, resulting in pangastritis in adulthood which is associated with mucosal atrophy and intestinal metaplasia which are precancerous conditions (Asaka et al., 1997, Atherton, 2006). It was therefore next hypothesised that the *H. pylori* acquisition might occur later in life. This might explain why a high prevalence may be associated with a low cancer incidence. Pangastritis and corpus-predominant gastritis are associated with increased risk of gastric cancer (Asaka et al., 1998, Asaka et al., 1997, Atherton, 2006, Blaser et al., 2007, Meining et al., 1998). Nothing is known about the distribution of *H. pylori*-associated inflammation in Iraqi patients in Kurdistan region, or its severity. Antral-predominant gastritis could explain the low cancer rate. Thus, we assessed the degree and distribution of inflammation in the stomachs of Iraqi people in this region.

H. pylori strains differ, and possession of specific virulence factors greatly increases the risk of disease. The best recognised of these are the *cag* pathogenicity island and active forms of the vacuolating cytotoxin VacA. *dupA* is a recently described gene shown to be associated with duodenal ulceration and protective

against gastric cancer (Lu et al., 2005a). In Iraq, no studies have yet been conducted to determine the prevalence of virulence factors of *H. pylori*, and their association with disease and histological findings. One objective of this project was therefore to investigate virulence factors of strains in a sample of patients undergoing upper GI endoscopy and any association with disease, and histopathological changes.

As described earlier, *H. pylori*–induced inflammation and Th1 responses are thought to be central to the pathogenesis of *H. pylori*–associated diseases. Environmental factors are known to influence the balance of the T-helper response. It was hypothesised that a Th2 response might predominate in Kurdistan region thus explaining the low gastric cancer incidence. Thus another aim of this project was to study the immune response to *H. pylori* and its association with virulence factors, clinical outcomes and other factors such as smoking and gender.

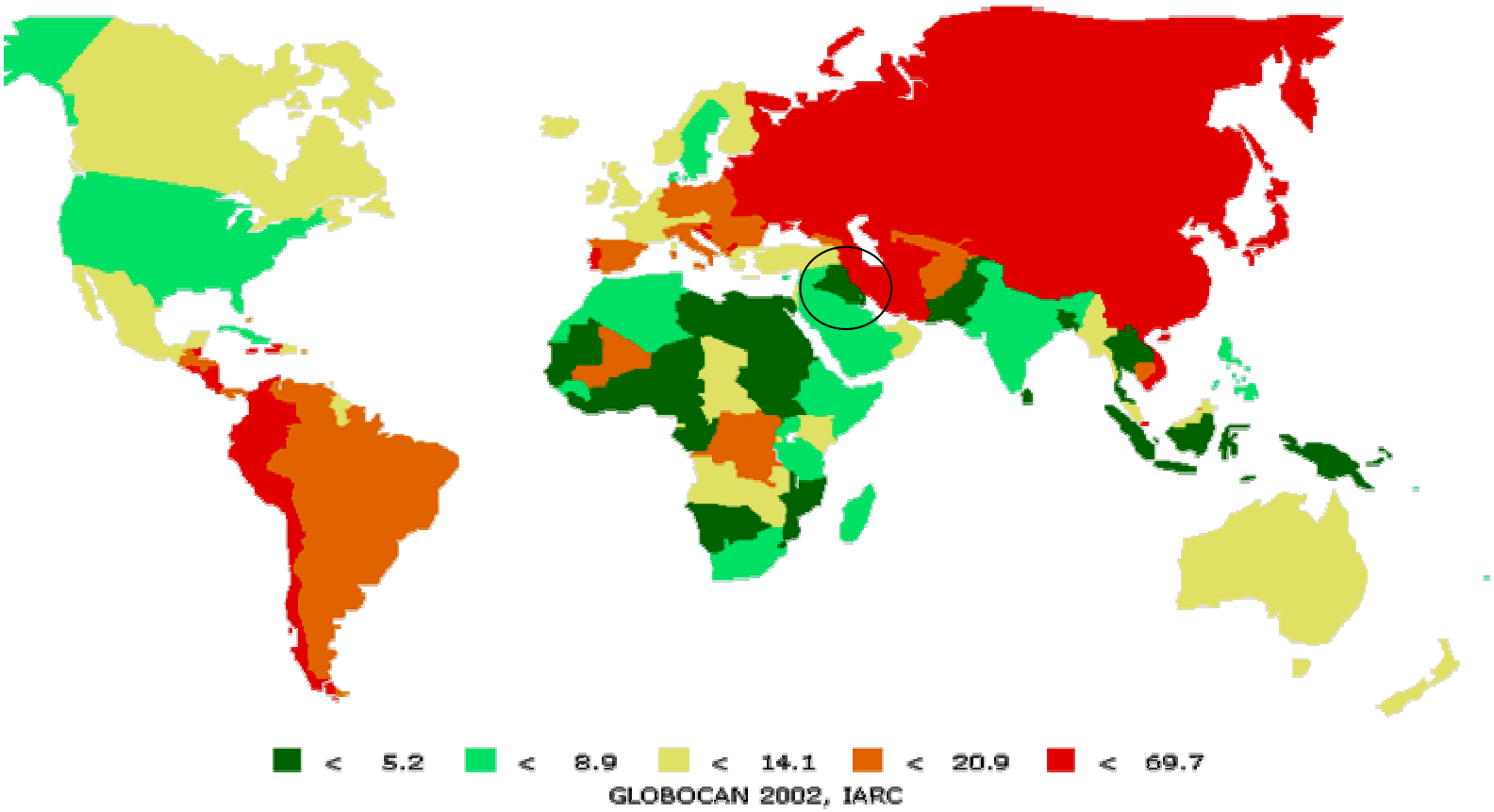

Figure 1. 3 Map showing worldwide gastric cancer rates (per 100,000)

2. Study of Age-Specific *Helicobacter pylori* Seropositivity Rates and *H. pylori*-Gastritis Patterns in Kurdistan region, Iraq.

Abstract

Introduction: In Iraq and especially Kurdistan region, gastric cancer is rare (5/100,000). To look for reasons for this, we investigated whether *Helicobacter pylori* infection was uncommon, whether it occurred late in life and whether infected adults had the antral-predominant pattern of gastritis which does not predispose to cancer. **Methods:** We determined age-specific *H. pylori* seropositivity rates by ELISA in 283 subjects from Dohuk city in Kurdistan and evaluated histopathological changes by Sydney system scoring in gastric biopsies taken from 30 *H. pylori*-infected adult subjects without peptic ulcers. **Results:** 79% of 163 adults were infected with *H. pylori*, while 37% of 120 children were seropositive ($p<0.0001$). The prevalence in children of various age group was <6months:0%; 6-24months:27%; 2-18years:58%. Amongst adults, the seroprevalence was similar across age groups and between sexes. For infected people, gastric lymphocyte infiltration was more prominent in the antrum ($p=0.01$). Neutrophil infiltration was mild. Histological evidence of mucosal atrophy was seen in only 1/30 antral and 1/30 corpus samples, and in these it was grade 1 (mild). **Conclusion:** *H. pylori* infection is common in Kurdistan and acquired at a young age. However, in adults, lymphocyte infiltration is more predominant in the antrum than the corpus. Furthermore, neutrophil infiltration is mild and gastric mucosal atrophy is rare. The mild pathology may help explain the low cancer rate in Kurdistan region. Further work is needed to understand why infection results in an antral-predominant gastritis with little atrophy.

2.1 Introduction

Helicobacter pylori is regarded as the most important risk factor for peptic ulcer disease and gastric cancer (Blaser et al., 1995, Correa, 1995, Correa, 2003). In Kurdistan region, northern Iraq, gastric cancer is rare (GLOBOCAN, 2002). Despite the geographical proximity of Iraq, Turkey and Iran the incidence of gastric cancer differs hugely amongst these countries; $\leq 5/10^5$, 8.9 to $14.1/10^5$ and 38 to $69/10^5$, respectively (GLOBOCAN, 2002, Nouraie et al., 2004, Sadjadi et al., 2003, Sadjadi et al., 2005, Yavari et al., 2006). One possible explanation for these differences would be similar differences in prevalence of *H. pylori* infection. However, data from Turkey and Iran do not support this idea: in Eastern Turkey, 64% of the population were found to be seropositive for *H. pylori* (Ayse SelimoGlu et al., 2002) whereas in Iran, *H. pylori* seroprevalence was only 33% (Nassrolahei and Khalilian, 2004). In another study from Iran, *H. pylori* seropositivity was 48% in Ardebil province and 31% in Yazd province (Mikaeli et al., 1999). Gastric cancer is more common in Ardebil than Yazd, so here differences in *H. pylori* prevalence may be contributing to the difference. The prevalence of *H. pylori* in Kurdistan region is unknown.

Diagnosis of *H. pylori* can be achieved by taking biopsies at endoscopy. However, this procedure is invasive and might not give accurate results if colonisation is patchy (Ormand and Talley., 1990). Furthermore, it does not suit population-based studies. For screening of populations, serodiagnosis remains the method of choice for measuring the prevalence of infection (Dooley et al., 1989). Systemic humoral immunoglobulin G (IgG) immune responses to the organism are developed by humans infected with *H. pylori* (Perez-Perez et al., 1988, Talley et al., 1991a,

Veenendaal et al., 1991). Serological tests are useful tools for the diagnosis of *H. pylori* infection because all *H. pylori*-infected patients produce an antibody response which can be detected in the serum (Perez-Perez et al., 1988). The technique of choice is currently ELISA because it is a simple, quick, and low-cost (Perez-Perez et al., 1988).

Previous reports have shown that patients infected at a younger age have an increased risk of later developing gastric cancer (Asaka et al., 1998, Asaka et al., 1997, Atherton, 2006, Blaser et al., 2007, Sepulveda et al., 2002). Early acquisition is associated with pangastritis in adulthood followed by gradual mucosal atrophy, intestinal metaplasia, dysplasia and gastric cancer (Asaka et al., 1998, Asaka et al., 1997, Atherton, 2006, Blaser et al., 2007, Meining et al., 1998, Sepulveda et al., 2002). Consistent with this, in Iran, *H. pylori* infection was shown to be significantly more prevalent amongst individuals of less than 20 years of age in areas with high incidence of gastric cancer compared with areas of low cancer incidence (Mikaeli et al., 1999). If infection occurred later in life in Iraq, this could potentially explain the low cancer rate. We investigated *H. pylori* prevalence in different age groups in children and in adults.

Pangastritis and corpus-predominant gastritis are associated with increased risk of gastric mucosal atrophy and increased risk of cancer (Asaka et al., 1998, Asaka et al., 1997, Atherton, 2006, Blaser et al., 2007, Meining et al., 1998). Nothing is known about the distribution of *H. pylori*-associated inflammation in Iraqi patients, or its severity. Potentially, the low cancer rate in Kurdistan region could be

explained by antral-predominant gastritis being the common pattern and/or by inflammation being mild.

Thus, we now aimed to assess *H. pylori* seroprevalence in children and adults in Kurdistan region in northern Iraq and the degree and distribution of inflammation in the stomachs of Iraqi people.

2.2 Materials and Methods

2.2.1 Patient-Derived Samples

Serum samples from 283 subjects including 120 children and 163 adults admitted to the surgical and pediatric wards for conditions other than gastrointestinal disorders were used in the study.

Gastric biopsies were obtained from dyspeptic patients undergoing routine upper gastrointestinal endoscopy in Azadi hospital/ Dohuk city, Kurdistan region, Iraq and found not to have peptic ulcer disease. A total of 35 *H. pylori*-infected adults (age: 17-70 years) underwent upper gastrointestinal endoscopy using a GIF XQ 40 endoscope (Olympus Optical Company, Tokyo, Japan) under topical lignocaine anaesthesia. During gastroscopy, two biopsies from the antrum and two from the corpus were taken and fixed in 10 ml buffered 10% formalin for histopathological examinations.

The study protocol was approved by the Ethics and Research Committees of the hospital, and all patients gave informed consent to the study.

2.2.2 Culture

Each biopsy specimen was spread on both sheep blood and Dent agar plates, then incubated under microaerobic conditions generated by a Campypack (Becton, Dickinson and company) in an anaerobic jar at 37°C for 2 to 4 days. The organisms were identified as *H. pylori* by colony morphology; Gram stain; and urease activity. Cultures were harvested and stored in 1 ml nutrient broth containing 15% glycerol at - 80°C.

2.2.3 Antigen Preparation for ELISA

A lysate of 5 Iraqi stains (all of which were *cagA*-positive) was prepared. The lysate was prepared by sonicating a suspension of a pool of the 5 Iraqi strains in PBS. This procedure was performed on ice using 6 cycles of 30 seconds, followed by 30 seconds cooling. The protein concentration was determined using a modified Lowry protein assay kit (Bio-Rad).

2.2.5 Serum IgG subset ELISA

As described previously (Robinson et al., 1997), Wells of a 96-well ELISA plate (NUNC maxisorp) were coated with 50 µl *H. pylori* sonicate antigen diluted to 3ng/ml in 0.1M carbonate/bicarbonate coating buffer (pH 9.6). The plates were then incubated overnight at 4°C. 100 µl of blocking solution (3% bovine serum albumin in PBS-Tween) was added to each well. Sera were tested in a twofold dilution series, including replicate wells of a 1/50 dilution of a pool of negative control sera from 4 uninfected donors on every plate. 50 µl of diluted (1/50,000) anti-human IgG-HRP (Sigma A8667) was added before development using Tetra methylbenzidene substrate (eBioscience). The optical density of the samples was measured using a microplate reader (Labsystems iEMS Reader MF) at a wavelength of 450nm with a reference wavelength of 650nm. Then, curves of dilution against mean OD for each sample were plotted (Figure 2.1).

2.2.4 Validation of the ELISA

To validate our results, a commercial kit (Biohit Plc) was used to do the ELISA for 25 randomly selected samples as per manufacturer's instructions.

2.2.6 Histological Examination

Paraffin-embedded biopsies were cut (4 µm thick) and stained with hematoxylin and eosin (Anim et al., 2000). The histological findings from the sections were scored according to the updated Sydney system of classification and grading of gastritis(Price, 1999). Slides were examined microscopically (Olympus Optical Company, Tokyo, Japan) using X40 magnification by a single pathologist. These slides were graded for the following features: *H. pylori* density, neutrophilic activity, lymphocytic infiltration, and glandular atrophy. A visual analogue scale was used to assess the severity of the inflammatory changes and grading was done as follows: l: mild, 2: moderate and 3: severe.

2.2.7 Statistics

Data were analyzed using the Minitab 15 software program. The Chi squared test was performed to study the difference in positivity rate between age groups. The Mann-Whitney test was used for the comparison of histopathological data. Spearman's test was used to study the relationship between age and histopathological changes. Differences were considered statistically significant at a p-value of less than 0.05.

2.3 Results

2.3.1 ELISA Assay Validation

Current commercial ELISA kits are based on antigens extracted from diverse *H. pylori* strains but we were unsure whether these included strains from the Middle East. Since our study involved assaying serum samples from Iraq, we decided to develop our own ELISA and use antigens prepared from strains in the local community. In addition, commercial kits measure anti-*H. pylori* IgG from one dilution of serum and compare this with a standard curve. We therefore designed an assay based upon a serial dilution method to give an antibody titre. This approach provides information on the level and the specificity of the response.

The assay cutoff score for positivity was determined by calculating the mean response of 5 sets of *H. pylori* negative sera (negative by CLO test) at 1/50 dilution plus 3 standard deviations. Any titre above that was regarded as seropositive (Figure 2.1). For the commercial kit, the cutoff scores for a positive result were based on the recommended values provided by the manufacturers. The values which were seropositive by the commercial kit were all positive by our ELISA and the values which were negative by the commercial kit were all negative by our ELISA, giving accuracy for our ELISA of 100%, when the commercial kit was used as gold standard (Figure 2.2).

In further support of our ELISA, we found that the distribution of anti-*H. pylori* IgG titres was bimodal. One group had values less than 500; this included all those who were *H. pylori* negative by the commercial kit. No patient had a titre between

500 and 2000. The titres of all the others were more than 2000, including all those that were seropositive by the commercial kit (Figure 2.2 and 2.3).

2.3.2 Sero-prevalence

The prevalence of *H. pylori* in children of different ages varied (age: percentage, 6months:0%; 6-24months:27%; 2-18years:58%) with an overall prevalence of *H. pylori* in children of 37% (44/120). 79% (128/163) of adults were infected with *H. pylori*, a significantly higher proportion than children ($p<0.0001$). The prevalence of *H. pylori* increased markedly with age with the maximum colonization (81.5%) occurring in adults (18-40years) (Figure 2.4). 63% (97/153) of males were infected with *H. pylori*, while 58% (76/130) of females were *H. pylori*-seropositive (p=ns).

2.3.3 Histological Analysis

Samples for histopathology were obtained from 35 adult subjects with dyspepsia and without peptic ulceration. Samples taken from one subject were considered inadequate for histopathology. Four subjects were negative for *H. pylori*. These 30 *H. pylori*-positive samples were available for detailed histopathology.

Pathological changes were observed in biopsies from 29/30 patients; biopsies from 1/30 were histologically normal despite *H. pylori* being cultured. Amongst the 29 patients with inflammation in gastric biopsies, lymphocyte infiltration was more prominent in the antrum (Mann Whitney U test p=0.01, Table 2.1). There was no significant difference in *H. pylori* density, neutrophil infiltration, or mucosal atrophy between antrum and corpus (Table 2.1). However, neutrophil infiltration was mild, and was virtually absent (score 0) in 41% antral biopsies and 59% corpus

biopsies. Furthermore, histological evidence of mucosal atrophy was seen in only 1/30 antral biopsies (patient age=40 years) and 1/30 corpus biopsies (patient age=64 years); in both cases it was mild. No association could be found between age and histopathological changes including atrophy and metaplasia.

Table 2. 1 *H. pylori*-associated gastritis in biopsies taken from gastric antrum and body. * Significant p value measured by Mann Whitney U test

Variables	Antrum				Corpus				
	Score 0	Score 1	Score 2	Score 3	Score 0	Score 1	Score 2	Score 3	p-value
Lymphocyte infiltration (n)	0	10	14	5	0	20	7	2	0.0164*
Activity of inflammation (n)	12	15	2	0	17	11	1	0	0.191
Intestinal metaplasia (n)	27	1	1	0	26	3	0	0	0.6883
Atrophy (n)	28	1	0	0	28	1	0	0	1

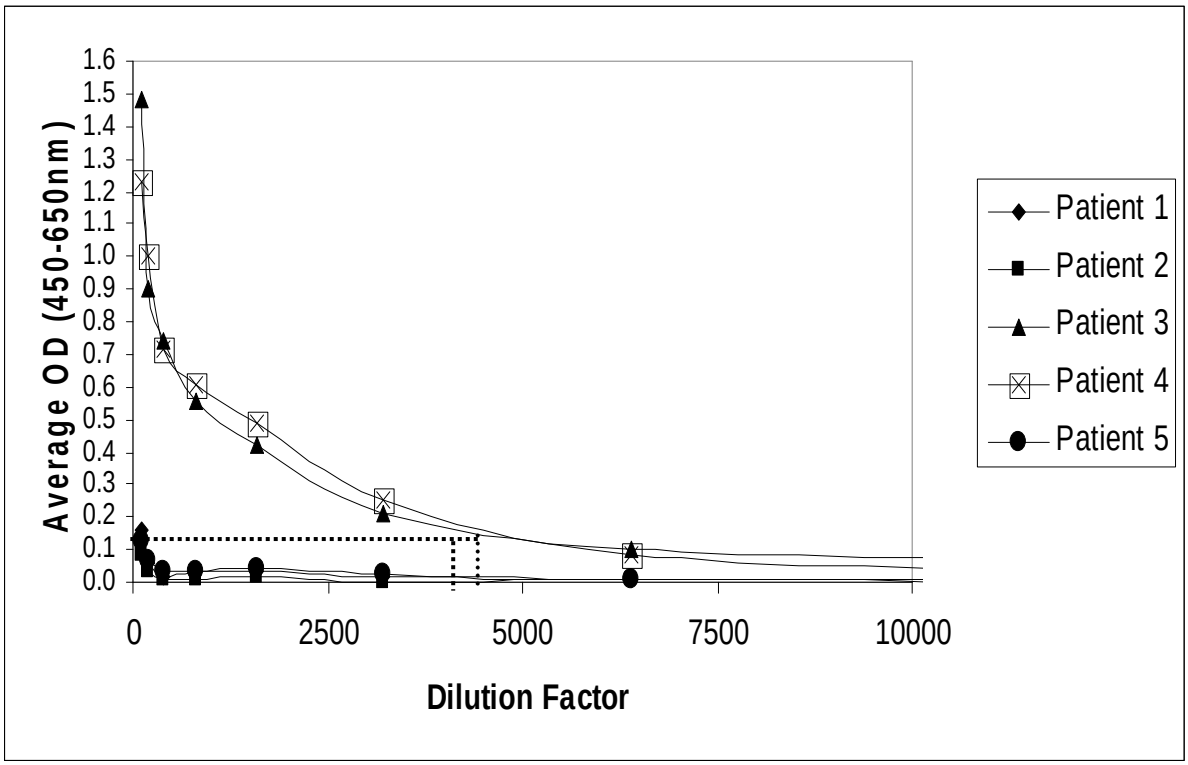

Figure 2. 1 An example of multiple dilution series showing the optical density measured by anti-human IgG ELISA and the corresponding dilution factor of 5 subjects.

The dilution titre for patient 3 was 4000 and for patient 4 was 4500. The average ODs for patient 1, 2 and 5 were around 100. The broken line indicates the OD cutoff (the mean response of 5 sets of *H. pylori* negative sera at 1/50 dilution plus 3 standard deviations).

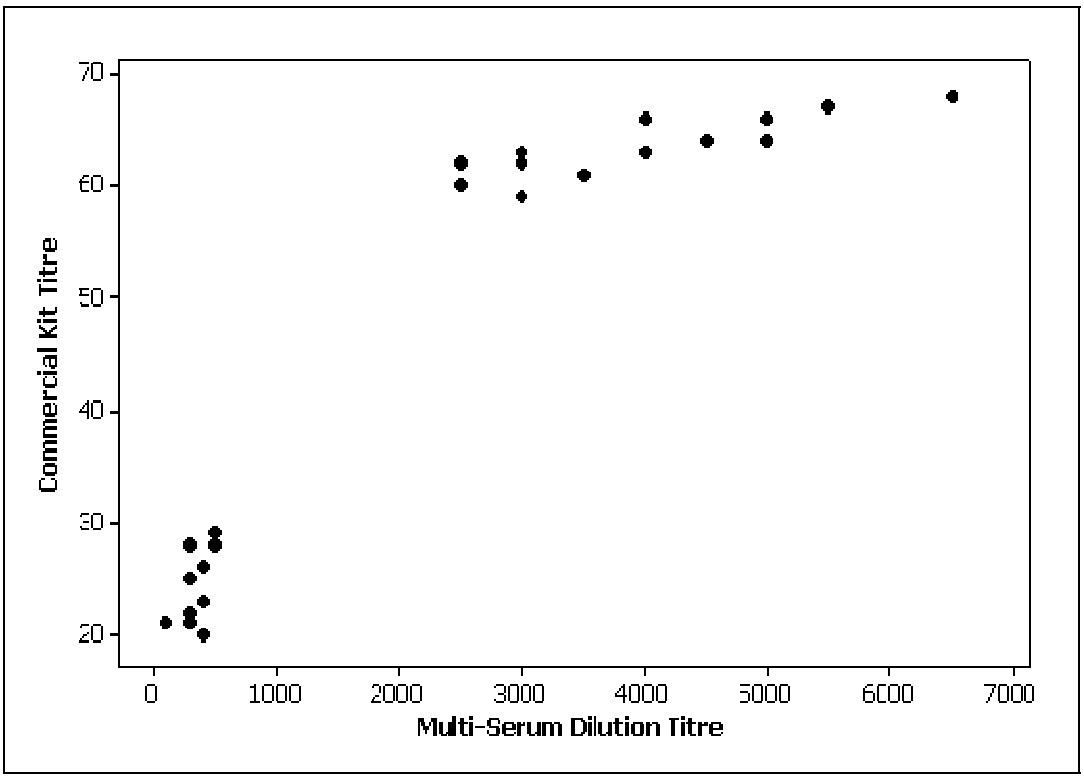

Figure 2. 2 Correlation between the multi-serum dilution titres of anti-human IgG and the titres of the commercial kit for 25 Kurdish Iraqi subjects

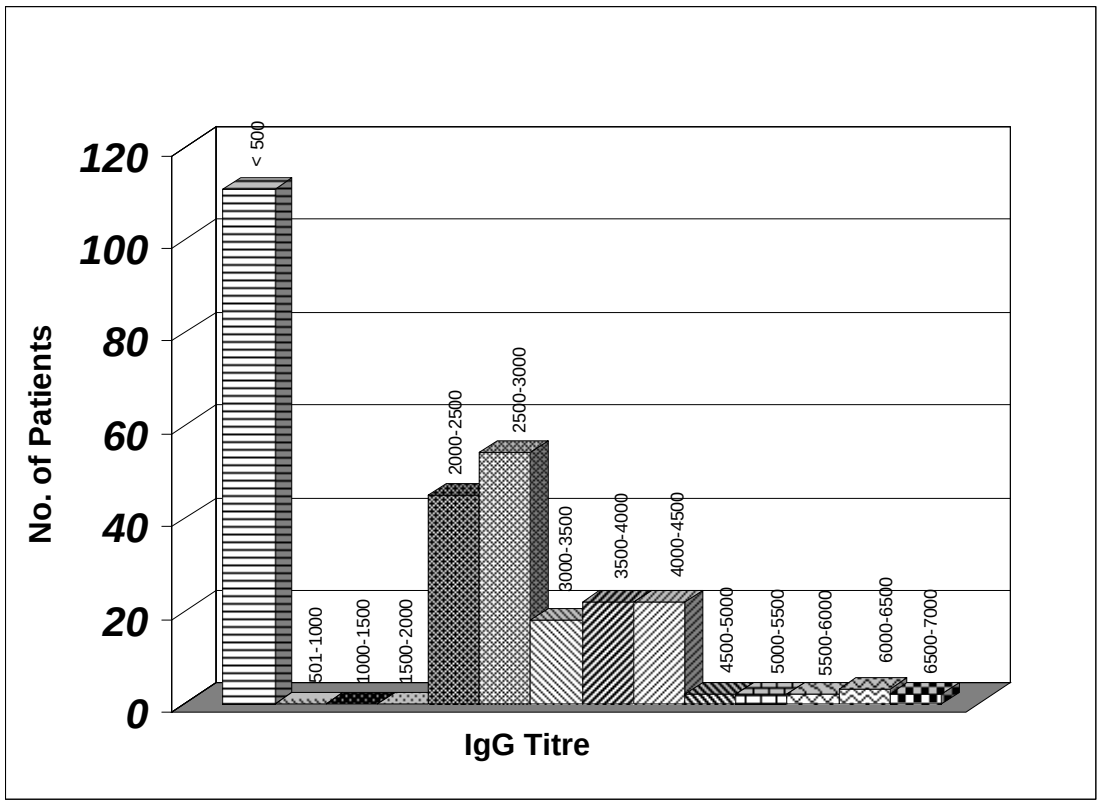

Figure 2. 3 Histogram showing the distribution of anti-*H. pylori* IgG in Kurdish Iraqi subjects.

The titre of all *H. pylori*-negative subjects is < 500 ng/ml. Notice that there is a window between titres for *H. pylori*-negative patients and *H. pylori*-positive patients.

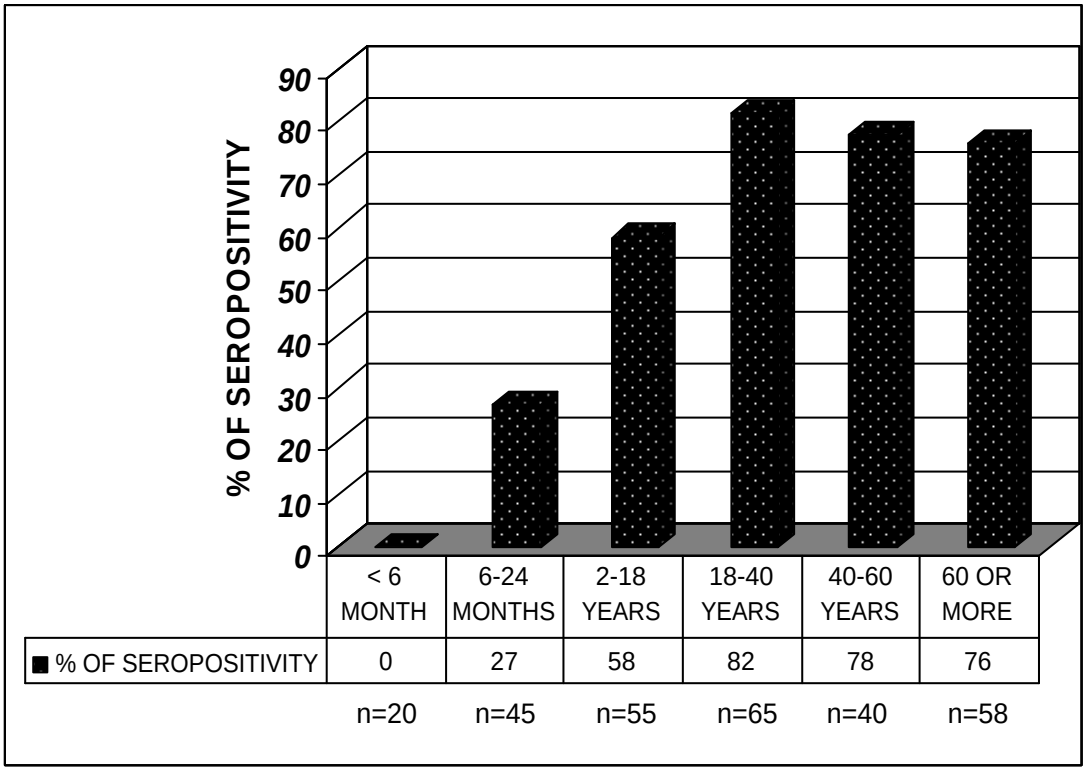

Figure 2. 4 The seropositivity of *Helicobacter pylori* infection in relation to age.

2.4 Discussion

Although *H. pylori* organisms have been isolated worldwide, they are more frequently recovered from patients in developing countries (Atherton, 2006). Our cross-sectional population-based serosurvey demonstrated that the prevalence of *H. pylori* infection among hospitalised patients in Dohuk city was 61%. The seropositivity was not related to gender. In agreement with other studies (Al-Moagel et al., 1990, Staat et al., 1996) a significant increase in the seroprevalence of *H. pylori* infection among asymptomatic children by age was found. The prevalence of *H. pylori* infection in Kurdistan region, Iraq is higher than reported in Iran (33%) (Nassrolahei and Khalilian, 2004) where the gastric cancer rate is very high and lower than Turkey (64.4%) where the gastric cancer rate is intermediate (GLOBOCAN, 2002). Thus, there is not a simple positive association between *H. pylori* and gastric cancer rate in these three countries, and the lower gastric cancer rate in Iraq is not due to low prevalence of *H. pylori*.

Asaka *et al.* argued that acquiring *H. pylori* infection at an early age usually results in pangastritis in adulthood. This pattern of gastritis in adults is often associated with mucosal atrophy - a precancerous condition (Asaka et al., 1997, Atherton, 2006). In support of this, early acquisition of *H. pylori* in childhood is a recognised risk factor for the development of gastric cancer (Asaka et al., 1997, Atherton, 2006, Blaser et al., 2007). The acquisition of *H. pylori* occurs in early childhood in most developing countries (Neale and Logan, 1995). According to our IgG based ELISA, none of the under 6 month children were found to be seropositive. These might be false negative results as the immune system in children of this age is not fully mature. However, 27% of the subjects aged 6 months-2 years were

seropositive in our assay and so appear to have acquired the infection. Unfortunately, we do not have individual ages of children in the 2-18 year age group, so we cannot comment further on the most common period of infections between these ages. However, it is clear from our results that at least a quarter of children are infected very early in life, and most infections in Kurdistan occur during childhood. Thus our study suggests that the low cancer rate in Kurdistan is unlikely to be because *H. pylori* is acquired late in life.

Greater inflammation of the gastric corpus compared with the antrum results in a greater risk of development of gastric adenocarcinoma (Meining et al., 1998). In a study conducted in Iran, where the gastric cancer rate is very high (GLOBOCAN, 2002), it was found that mononuclear cell infiltration was similar throughout the stomach; on average, patients had pangastritis (Sotoudeh et al., 2008). In Kurdistan region, Iraq, we have shown that there is antral-predominant mononuclear cell infiltration. This could partially contribute to the difference in cancer rate between these two countries. Furthermore, in Iran, histological evidence of mucosal atrophy was seen in 39% and 22% of antral and corpus samples, respectively (Malekzadeh et al., 2004). In another study conducted in another province in Iran (where only antral biopsies were examined) atrophic changes was found in 25% (Hashemi et al., 2006). In another study conducted in Turkey, it was found that 43% of the *H. pylori*-infected subjects had atrophic gastritis (Fikret et al., 2001). In our study, glandular atrophy was found in only one (3%) specimen taken from antrum and one from the corpus. Thus despite the early acquisition of *H. pylori*, the presence of atrophy appears rare in this region of Iraq. We speculate that this might contribute to the low cancer rate. Why gastric mucosal atrophy is uncommon in Kurdistan

needs further study. However, our results raise the possibility that it may in part be due to the antral-predominant infiltration pattern seen in *H. pylori* infected Kurdish people.

Our study has limitations; in particular the sample size used for our histopathology study is small. However, because of the low rate of atrophy and intestinal metaplasia it is unlikely to be misleading.

To conclude, *H. pylori* infection is common in Kurdistan and acquired at young age. However, in adults lymphocyte infiltration is more predominant in the antrum than the corpus (an antral predominant gastritis). Furthermore, gastric mucosal atrophy is uncommon. This may help explain the low cancer rate in Kurdistan, particularly when compared with Iran. Further work is now needed to study bacterial virulence factors, host genetics and environmental factors in this region of Iraq, to understand why infection results in an antral predominant gastritis with little atrophy.

3. Differences in Virulence Markers between *Helicobacter pylori* Strains from Kurdistan Region (Iraq) and Iran: Potential Importance for Regional Differences in *H. pylori*-associated Disease.

Abstract

Helicobacter pylori causes peptic ulceration and gastric adenocarcinoma; the latter is common in Iran but not Iraq. We hypothesised that more virulent *H. pylori* strains may be found in Iran than Iraq and so compared established and newly-described virulence factors in strains from these countries. We studied 59 unselected dyspeptic patients from Iran and 49 from Kurdistan region, Iraq. *cagA* was found in similar proportions of strains (Iran 76% vs Iraq 71%) and was significantly associated with peptic ulcer disease (PUD) in Kurdistan region, Iraq ($p<0.01$) but not Iran. *cagA* alleles encoding four or more tyrosine phosphorylation motifs were found in 12% Iranian but no Iraqi (Kurdish) strains ($p=0.02$). There were no significant differences in *vacA* signal, mid or intermediate region types between Iranian and Iraqi strains. In Iran, *vacA* genotypes showed no specific peptic ulcer associations, but in Kurdistan *vacA* i1 strains were associated with gastric ulcer ($p<0.02$), mimicking their previously demonstrated association with gastric cancer in Iran. *dupA* was found in similar proportions of Iranian and Iraqi strains (38% vs 32%) and was associated with peptic ulceration in Iraqi ($p<0.01$) but not Iranian patients. *H. pylori* strains from Iraq and Iran possess virulence factors similar to those in western countries. The presence of *cagA* with more phosphorylation motifs in Iranian strains may contribute to the higher incidence of gastric cancer. However, the association between strain virulence markers and disease in Iraq but not Iran suggests that other host and environmental factors may be more important in the disease-prone Iranian population.

3.1 Introduction

Helicobacter pylori is a spiral-shaped, Gram-negative bacillus which causes gastritis and peptic ulceration (Marshall and Warren, 1984, Marshall and Warren, 2001, Whitfield, 2003). Its treatment has become pivotal in the management of peptic ulcer disease. *H. pylori* infection is also an important risk factor for gastric adenocarcinoma, the second highest cause of cancer deaths worldwide. Gastric cancer is thought to have a multifactorial etiology, and bacterial strain type, host genotype, and environmental conditions, are all thought to contribute (Peek and Blaser, 2002). Despite the geographical proximity of Iraq and Iran the incidence of gastric cancer differs hughly between these countries; in Iran it ranges from 38-69/10^5 (GLOBOCAN, 2002, Nouraie et al., 2004, Sadjadi et al., 2003, Sadjadi et al., 2005, Yavari et al., 2006) compared to 5/10^5 in Iraq (GLOBOCAN, 2002). We hypothesised that this difference may be due to differences in virulence of circulating *H. pylori* strains and so set out to type strains from these countries for virulence. We considered both well-established and more recently-described virulence determinants.

Many strains of *H. pylori* produce the CagA protein, encoded by the *cagA* gene within the *cag* pathogenicity island (PAI). *H. pylori* strains possessing *cagA* are associated with a significantly increased risk for the development of atrophic gastritis, peptic ulcer disease (PUD) and gastric cancer (Rokkas et al., 1999, Tomb et al., 1997). The *cag* PAI encodes a type IV secretion system that facilitates the translocation of CagA into the host epithelial cytosol where it becomes tyrosine phosphorylated at specific phosphorylation motifs by Src family kinases (Selbach et al., 2002, Stein et al., 2002). Phosphorylated CagA forms a physical complex

with SHP-2 phosphatase and stimulates cell signalling pathways, cytoskeletal changes and abnormal cell proliferation (Tsutsumi et al., 2003). Based on the amino acid sequence of the SHP-2 binding site, CagA proteins can be sub-categorised into Western and East Asian types. Both have type A and B phosphorylation motifs (usually one of each) but western types have additional C motifs (1-3) and the East Asian type has no C motifs but a D motif. The East Asian type CagA possesses stronger SHP-2 binding and transforming activities than the Western-type CagA (Higashi et al., 2002a). Western-type CagA has a variable number of type C phosphorylation motifs, and the extent of cytoskeletal changes induced by CagA is dependent on this. Strains possessing CagA with greater numbers of type C phosphorylation motifs are more closely associated with gastric carcinogenesis (Azuma et al., 2002). Thus, determination of the degree of CagA phosphorylation or the number of phosphorylation motifs appears to be more important than detection of *cagA* alone (Argent et al., 2004, Argent et al., 2005)

The vacuolating cytotoxin (VacA) is a well-established *H. pylori* virulence factor which has multiple effects including vacuolation of cultured epithelial cells, inducing apoptosis, increasing permability of epithelial monolayers, forming pores in cells and suppressing immune cell function (Atherton, 2006, Iwamoto et al., 1999). The *vacA* gene is polymorphic within its signal, intermediate and mid regions. For the signal region, two distinct allelic sequences have been recognised, s1 and s2. For the mid region, alleles can be categorised into two classes, m1 or m2. The *vacA* genotype is associated with *in vitro* cytotoxin activity (s1> s2 and m1> m2) (Atherton, 2006). Rhead *et al* have recently described a novel determinant of VacA toxcity, the intermediate or i-region (Joanne et al., 2007). They showed that

two allelic variants of this region exist, i1 and i2. Furthermore, they showed that only s1/m2 strains varied in i-type; s1/m1 and s2/m2 strains were exclusively i1 and i2, respectively. This novel region determines vacuolating activity among these s1/m2 strains. Most importantly, a significant correlation was found between i1 type *vacA* and gastric cancer in Iran (Joanne et al., 2007).

The duodenal ulcer promoting gene A (*dupA*) is a recently described virulence factor which comprises both *jhp0917* and *jhp0918* (Lu et al., 2005a). Lu *et al.* found a significant relationship between *dupA* and DU, and the presence of *dupA* was related to neutrophil infiltration and a high level of IL-8 production by epithelial cells. Surprisingly, possession of this gene appeared protective against gastric adenocarcinoma (Lu et al., 2005a).

The object of this study was to type the virulence of unselected strains from dyspeptic patients in Iran and Iraq (Kurdistan region). We aimed to compare virulence of strains from these neighbouring countries with very different gastric cancer incidences and to assess the association of virulence markers with peptic ulcer disease in each country.

3.2 Materials and Methods

3.2.1 Patient-derived Samples

Gastric biopsies were obtained from 49 and 59 unselected *H. pylori*+ patients from Iraq and Iran respectively undergoing routine upper GI endoscopy to investigate dyspepsia. Mean age ± standard deviation of Iraqi patients was 35±17 years and of Iranian patients was 40±14 years. All Iraqi patients were from the 5 districts of Dohuk city, Kurdistan region. The majority (48/59, 81%) of Iranian patients were from Tehran city; others were referred from different regions in Iran. Endoscopic diagnoses were: duodenal ulcer (DU) Iraq 15, Iran 8; gastric ulcer (GU) 5, 9; no ulcer (NUD) 29, 42. During gastroscopy, biopsy samples were taken and either placed in 1 ml of isosensitest broth (Oxoid, Basingstoke UK) containing 15% (v/v) glycerol and stored in liquid nitrogen or cultured immediately for *H. pylori*. In some cases, following prolonged storage and shipping to the UK, re-culture was not possible. In these cases, DNA was extracted directly from the biopsy specimens and used for PCR-based *H. pylori* typing.

The study protocol was approved by the Ethics and Research Committees of the individual hospitals and all patients gave informed consent to the study.

3.2.2 Culture

Each biopsy specimen was spread onto horse blood or Dent agar plates, then incubated under microaerobic conditions generated by a CampyPak (Becton Dickinson, Baltimore, USA) in an anaerobic jar at 37°C for 2 to 4 days. The organisms were identified as *H. pylori* by colony morphology, Gram stain, and

urease activity. Cultures were harvested as sweeps rather than single colonies and stored in isosensitest broth containing 15% (v/v) glycerol at -80°C.

3.2.3 DNA Isolation from Bacteria

Two methods were used to extract genomic DNA. DNeasy tissue kits (QIAGENE) was used to extract DNA from biopsies following the manufacturer instructions.

In the second method, genomic DNA was prepared from confluent blood agar plate cultures. Bacteria were harvested and suspended in 500 µl PBS. The suspension was then centrifuged at 13000g for 5 min. The supernatant was discarded and the pellet was resuspended in TE buffer (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA). Then 500 µl GES (60g guanidine HCl, 20ml EDTA, pH 8.0, and 10% 5 ml sarcosyl) was added and the mixture was incubated at room temperature for 5 min. Following the addition of 250 µl of ice cold 7M ammonium acetate, the mixture was incubated on ice for 5 min. This was followed by adding 600 µl chloroform and the mixture was centrifuged at 13000g for 5-10 min. The top aqueous layer containing DNA was removed into a fresh tube using cut end tips. Chloroform step was performed twice, and the DNA precipitated from the aqueous phase with 0.54 volume of propan-2-ol. The DNA- propan-2-ol mixture was centrifuged at 13000g for 5 min. The supernatant was discarded and the pellet washed twice with 70% 500µl ethanol. The pellet was resuspended in 100µl TE buffer, and stored at 4°C.

3.2.4 Genotyping of *H. pylori*

Thermal cycling for amplifying *cagA* was 95 °C for 30 s, 50 °C for 1 min , and 72 °C for 2 min, for a total of 35 cycles. PCR amplification of *cagA* used previously described primers cag2 (5′ GGAACCCTAGTCGGTAATG 3′) and cag4 (5′ ATCTTTGAGCTTGTCTATCG 3′) to amplify about 500-bp product from the middle of *cagA* (PAI) (Rudi et al., 1998). PCR amplification of the *cag* PAI empty site was performed as previously described (Akopyants et al., 1998). In the empty site PCR, primers anneal to sequences adjacent to the *cag* PAI insertion site in the genome and only allow amplification of a DNA fragment of the expected size in the absence of a complete or partial *cag* PAI at this locus. Determination of the number of CagA phosphorylation motifs, and the types of motifs, was carried out by using the forward primer cag2 and the reverse primers *cagA*-P1C, *cagA*-P2CG, *cagA*-P2TA (the B motif is polymorphic: the two reverse primers are designed to recognise all types described to date), and *cagA*-P3E as previously described (Argent et al., 2005) (Figure 3.2).

For *vacA* signal and middle region, thermal cycle conditions were 30 s at 95°C, 60 s at 56°C, and 90 s at 72°C were performed for 35 cycles. Primers used for the middle region were vagF (5′ CAATCTGTCCAATCAAGCGAG 3′), and vagR (5′ GCGTCTAAATAATTCCAAGG 3′). For the signal region, A3436 (5′ ATGGAAATACAACAAACACAC 3′) and C1226 (5′ CTGCTTGAATGCGCCAAAC 3′) primers were used (Atherton et al., 1995).

The PCR conditions for *vacA* i region were as follows: the template mixtures were amplified 35 cycles with primers VacF1 (5′ GTTGGGATTGGGGGAATGCCG 3′)

and C1R (5′ TTAATTTAACGCTGTTTGAAG 3′) for i1 region and VacF1 and C2R (5′ GATCAACGCTCTGATTTGA 3′) for i2 region, at 95 °C for 30 s, 53 °C for 1 min, and 72°C for 30 s (Joanne et al., 2007).

The *dupA* was amplified by using standard protocols with the following block cycler conditions: 35 cycles, each consisting of denaturation at 95°C for 30 s, annealing at 50°C for 1 min, and elongation at 72°C for 2 min. 4 primers were used DupAF113 (5′ GACGATTGAGCGATGGGAATAT 3′), DupAR1083 (5′ CTGAGAAGCCTTATTATCTTGTTGG 3′), DupAF1202 (5′ TAAAATCACAAGGGGAAAAGATC 3′) and DupA918R (5′ AAGCTGAAGCGTTTGTAACG 3′) (Argent et al., 2007, Lu et al., 2005a) (Figure 3.2).

Amplification of above mentioned genes started with an initial denaturation at 95°C for 30 s and a final elongation step of 5 min at 72°C. Reactions were performed in 25 µl volume. 1 µl of *H. pylori* genomic DNA, 1 µl primer, 0.5 µl of Taq DNA polymerase, 0.5 µl dNTP, and 2.5 10X PCR buffer.

5µl of the PCR products were electrophoresed in 1.5% agarose gels for others for 40 minutes at 80 V in 1X TAE buffer. All gels were stained with ethidium bromide (1 mg/l) and photographed under UV light. 1 kb DNA ladder (Gibco, Paisley, UK) was used as a size marker (M) in all gels.

The resulting *H. pylori* strains *vacA* genotypes compared with 8823 (s1/i1/m1) and Tx30a (s2/i2/m2) lab strains. For *cagA* and *dupA*, 8823 and J99 were used as a positive control respectively.

3.2.5 Data Analysis

Statistical analysis of data was performed by using logistic regression, chi-square test and Fisher's exact test with significance set at a p value of < 0.05. Genotypes with mixed status for *vacA* were excluded from the calculations of association.

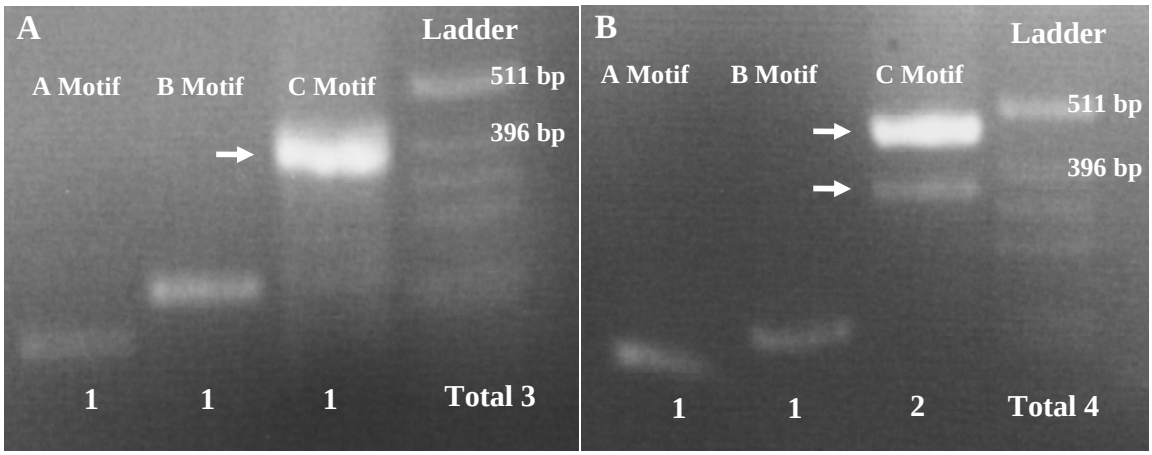

Figure 3. 1 Characterisation of one Kurdish Iraqi (A) and one Iranian (B) strain for *cagA* variable-region tyrosine phosphorylation motifs.

Genomic DNA samples from *H. pylori* strains were used to PCR amplify the *cagA* variable-region EPIYA motifs, using the forward primer cag2 and the reverse primers *cagA*-P1C (A motif), *cagA*-P2CG and *cagA*-P2TA (B motif), or *cagA*-P3E (C motif). No Iraqi strain was found to have more than 3 phosphorylation motifs.

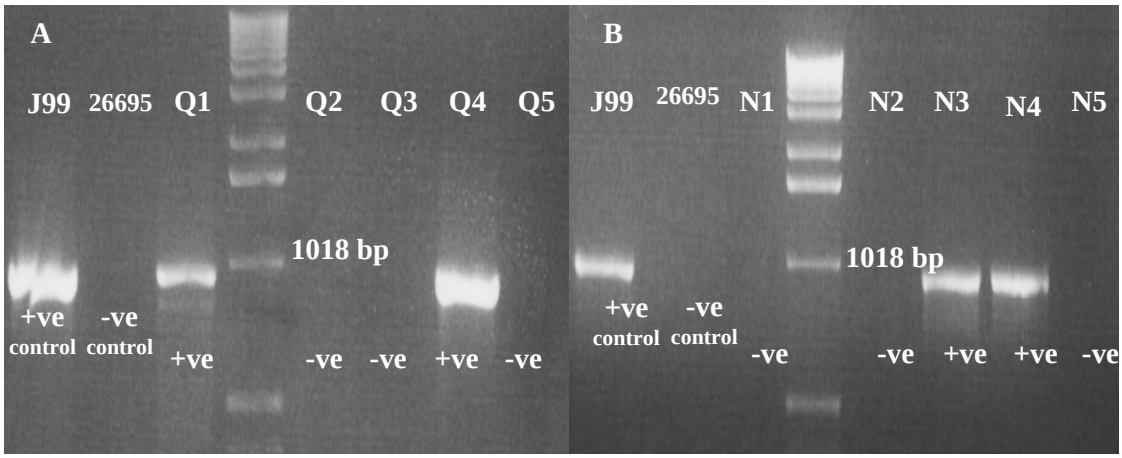

Figure 3. 2 Characterisation of Kurdish Iraqi (A) and Iranian (B) strains for *dupA* using PCR. Image shows results of PCR typing of *dupA* using primers DupAF113 and DupAR1083.

3.3 Results

3.3.1 Prevalence of *cagA*+ Strains among Dyspeptic Iranian and Iraqi (Kurdish) Populations

First, whether the prevalence of *cagA*+ strains was similar or different between Iraqi (Kurdish) and Iranian populations was assessed. Among unselected Iranian and Iraqi patients with dyspepsia, *cagA*+ strains were present in 76% (45/59) and 71% (35/49) *H. pylori* strains respectively (Table 3.1). To exclude bias from disease association, we also compared subgroups without peptic ulceration: *cagA* was found in a higher proportion of Iranian than Iraqi strains (76% vs 55%) although this did not quite achieve formal statistical significance (p=0.06). In both countries, all *cagA* positive strains also typed positive for *cagE*. Amongst 14 *cagA*-negative Iraqi strains, 7 were *cag* PAI empty site-positive (implying that the whole *cag* Pathogenicity Island was absent) and 7 were empty site negative (implying that there was still a partial *cag* PaI at this locus). Among 14 *cagA*-negative Iranian strains, 9 were *cag* PAI empty site-negative. Thus overall, the *cag* PAI appeared to be incompletely deleted in 16 strains.

No significant association was found between *cagA* status and clinical outcome in Iranian patients, but a significant correlation was found between *cagA* and peptic ulcer diseases ($p < 0.01$, OR 16.4**)** in Iraqi patients (Table 3.1). Considering duodenal and gastric ulcer separately in the Iraqi population, 14/15 (93%) patients with DU had *cagA*-positive strains, compared with 16/29 (55%) with no ulcer ($p < 0.02$). All Iraqi GU patients had *cagA*-positive strains (p= not significant compared with no ulcer, perhaps due to the low number of GU patients).

3.3.2 CagA Phosphorylation Motif Numbers

Next, *cagA* polymorphisms was studied, and in particular the number of CagA phosphorylation motifs, which we assessed using our recently-described PCR-based typing system (Argent et al., 2005). Among *cagA*+ strains, 12% (7/49) from Iran carried a *cagA* variable region >550bp (when amplified using primers cag2 and cag4) indicating the presence of more than 3 CagA phosphorylation motifs. This was a significantly higher proportion than found in Iraq, where all strains possessed *cagA* with a variable region size of 550bp indicating the presence of CagA with 3 phosphorylation motifs (p= 0.02) (Table 3.1). In the analysis confined to patients without ulcers, 22% (7/32) *cagA*+ Iranian strains had >3 phosphorylation sites compared with no Iraqi strains. Previous studies in other populations have linked multiple CagA phosphorylation motif numbers with increased cancer risk but not with increased ulcer risk (Azuma et al., 2002, Kidd et al., 1999): in agreement with this, no strains with > 3 phosphorylation motifs were found in the ulcer group in Iran (Table 3.1).

3.3.3 *vacA* Polymorphism

Then, *vacA* polymorphisms in Iranian and Iraqi strains were studied, examining both established s and m genotypes, and also the recently described polymorphic i region type. Since individual *H. pylori* isolates possess only a single copy of *vacA*, the presence of more than one *vacA* s, i, or m genotype in a DNA sample indicates colonisation by two or more strains with different *vacA* genotypes (Atherton et al., 1995). In Iraqi isolates, a single *vacA* signal region was observed in all samples, but 8/49 (16%) of the specimens examined possessed both mid region types, and 9/49 (18%) possessed both i region genotypes. In Iranian samples, all isolates possessed

a single signal region type, but 2/59 (3%) carried both m region types and 7/59 (12%) possessed both i region types. There was no difference in the prevalence of strains with different *vacA* genotypes in the unselected dyspeptic populations from Iran and Iraq, whether strains with multiple genotypes were excluded (planned analysis, Table 3.2) or classified as the more pathogenic or the less pathogenic type (exploratory analyses).

Next, the associations between *vacA* allelic variation and peptic ulceration within the Iranian and Iraqi populations were studied. For Iranian strains, no significant associations were found. For Iraqi strains, no significant association was found for duodenal ulceration, but 80% (4/5) strains isolated from gastric ulcer patients were of the *vacA* i1 genotype, significantly more than the 13% (4/29) strains from patients without ulcers ($p< 0.02$). This subgroup analysis, although exploratory, is interesting, given the described association between *vacA* i1 genotype and gastric cancer and the similarities in epidemiology and pathogenesis between gastric ulcer and gastric cancer. Associations were not seen between gastric ulceration and *vacA* s and m type, again supporting the recent finding that *vacA* i type is a better marker of strain virulence (Joanne et al., 2007)

3.3.4 *dupA* Status

Thirdly, I examined strains for the recently-described putative virulence gene *dupA*. Similar proportions of Iranian and Iraqi strains possessed *dupA* (Table 3.1). Among Iranian patients, no association between *dupA* and clinical outcome was found. However, among Iraqi patients, 55% (11/20) peptic ulcer patients carried *dupA*+ strains, significantly more than the 17% (5/29) non-ulcer patients ($p< 0.01$, OR 6.2,

Table 1). When I looked at DU and GU separately, 60% (9/15) *H. pylori* isolates from DU patients were *dupA*-positive (p< 0.01 *vs* non-ulcer patients) and 40% (2/5) *H. pylori* isolates from GU patients were *dupA*-positive (p= not significant *vs* non-ulcer patients, but note small number of GU patients).

3.3.5 Associations between Virulence Factors, Particularly for *cagA* Phosphorylation Motif Number

Next, associations between virulence factors in strains from Iran and Iraq were assessed. As in virtually all strain populations worldwide, I found *cagA*+ strains were more likely to be *vacA* s1 than s2: in Iran, 37/45 (82%) *cagA*+ strains were *vacA* s1 versus 5/14 (36%) *cagA*-negative strains (p< 0.005); in Iraq, all *cagA*+ strains typed s1 compared with 10/14 (71%) of *cagA*-negative strains (p<0.005). No significant associations were found between *cagA* status and other *vacA* polymorphisms or between *cagA* status and *dupA* status. As strains with larger *cagA* are thought to be more pathogenic than those with smaller *cagA*, I examined the association between the size of *cagA* and other virulence factors among Iranian strains. This analysis was not possible for Iraqi strains as all had the same number (3) of CagA phosphorylation motifs. Seven of 45 (15%) *cagA*+ Iranian strains carried a larger size *cagA* (with more than 3 phosphorylation motifs). In the association analysis with *vacA* genotypes, patients with mixed genotypes were excluded. The small numbers of strains studied meant that most associations were not statistically significant, but for the *vacA* m region, 6/7 (86%) strains with >3 motifs were type m1, significantly more than the 10/37 (27%) with 3 motifs only (p= 0.01). Lastly I examined the association between size of *cagA* and *dupA* status:

6/7 (86%) strains with >3 motifs were *dupA*+, significantly more than the 14/38 (36%) with 3 motifs only (p= 0.03).

Table 3. 1 *cagA* status, *cagA* phosphorylation motif number and *dupA* status among *H. pylori* strains from unselected Iranian and Kurdish Iraqi patients with dyspepsia.

	% (No.) positive for								
	cagA positive			> 3 *cagA* phosphorylation motifs strains			*dupA* positive		
	PUD	NPUD	Total	PUD	NPUD	Total	PUD	NPUD	Total
Iraq	95%(19/20)[a]	55% (16/29)	71% (35/49)	0% (0/20)	0% (0/29)	0% (0/49)	55%(11/20)[a]	17%(5/29)	32%(6/49)
Iran	76%(13/17)	76%(32/42)	76%(45/59)	0%(0/17)	17%(7/42)	12%(7/59)[b]	35%(6/17)	40%(17/42)	39%(23/59)

[a] $P < 0.05$ for comparison between PUD and NPUD

[b] The presence of *cagA* alleles with > 3 phosphorylation motifs was significantly higher among Iranian than Iraqi strains ($p< 0.01$)

Abbreviations: PUD, peptic ulcer disease, NPUD, no peptic ulcer disease.

Table 3. 2 Distribution of *vacA* allelic types among *H. pylori* strains isolated from unselected dyspeptic patients from Iraq (Kurdistan region) and Iran

	s1/i1/m1	**s1/i1/m2**	**s1/i2/m1**	**s1/i2/m2**	**s2/i2/m2**	**mixed**
Iraq	8/49(16.3%)	2/49(4.1%)	1/49(2.0%)	20/49(40.8%)	4/49(8.2%)	14/49(28.5%)
Iran	15/59(25.4%)	4/59(6.7%)	1/59(1.7%)	16/59(27.1%)	16/59(27.1%)	7/59(11.9%)

3.4 Discussion

H. pylori virulence factors are important to study in populations, as they contribute to disease risk. For example, in Japan, where gastric cancer is common, more than 90% of *H. pylori* strains are *cagA* positive (Maeda et al., 1998). The gastric cancer rate in Iraq is lower than that in Iran; I hypothesised that difference in virulence factors of *H. pylori* strains between these two countries may partially explain this difference. I found no difference in prevalence of *cagA*+ strains between unselected dyspeptic populations from these countries, although among patients without ulcers *cagA*+ strains were 21% more prevalent in Iran (p= 0.06). Furthermore, Iranian patients with *cagA*+ strains were more likely to have the more pathogenic forms of *cagA* encoding 4 or more tyrosine phosphorylation sites, and among patients without ulcers this difference was 27%. Taking these results together, this represents a considerable difference in potential *cagA*-associated pathogenicity which could contribute to the differences in gastric cancer rates seen between these communities: both *cagA* status and the number of *cagA* phosphorylation motifs have been linked with cancer prevalence in a number of populations (Argent et al., 2004, Higashi et al., 2002a). However, I found no significant differences between Iranian and Iraqi populations in *vacA* types, and in particular in the i region type which has recently been linked with gastric cancer risk in Iran (Joanne et al., 2007). Also I found no difference in *dupA* status, which I studied because *dupA* has been reported to have a negative association with gastric cancer (Lu et al., 2005a) although recent data from us dispute this (Argent et al., 2007).

In the present report, I have shown that 71% and 76% of *H. pylori* strains isolated from Iraqi and Iranian samples respectively were *cagA* positive. This figure is closer to Western countries and Turkey than East or South East Asia (Arents, 2001, Kersulyte et al., 2000, Saribasak H, 2004, Stephens et al., 1998). Thus it appears that the high cancer rate in Iran is not due to the presence of East Asian type strains in that country.

I looked within the Iranian and Iraqi populations for associations between virulence factors and peptic ulcer disease. Among Iraqi strains, but not Iranian strains, I observed an association between *cagA* positive status and PUD. Reports from a neighbouring country, Turkey, have shown similar results to Iraq (Saribasak H, 2004). No Iraqi strains had *cagA* with >3 phosphorylation motifs so I could not examine for disease associations here. The situation in Iran was interesting: no strains with >3 phosphorylation motifs were found in patients with peptic ulcer. This may imply that the presence of > 3 phosphorylation motifs is protective against ulcers rather than specifically predisposing to cancer, as previously reported (Argent et al., 2004, Higashi et al., 2002a). For *vacA* polymorphisms I found no association between *vacA* i region and clinical outcome in Iranian samples. However, in Kurdish Iraqi specimens, a novel association was found between *vacA* i1 strains and gastric ulcer. This is not unexpected as gastric ulcer and gastric cancer are epidemiologically similar. However, our results need confirmation in other populations as only small number of gastric ulcer patients were enrolled in this study. For *dupA,* a significant link with PUD was present in the Iraqi population, but no association was found in Iranians. Thus overall, I showed that the Iraqi population was similar to western populations in terms of the association

of many virulence factors with ulcer disease. In contrast, these associations were not seen in the Iranian population. This may imply that factors other than bacterial virulence are most important for ulcer risk in Iran.

Many previous reports have shown clustering of active virulence factors within *H. pylori* strains, for example associations between *cagA*+ status and the *vacA* s1 genotype (Xiang et al., 1995). In agreement with these reports, I found a significant correlation between *cagA*+ status and *vacA* s1 genotype in Iran and Iraq. In addition, I showed a significant association between greater number of *cagA* phosphorylation motifs and both the *vacA* m1 genotype and *dupA*+ status. This further supports the concept of clustering of virulence factors, such that the majority of *H. pylori* strains possess either many or few, and that it is favourable for *H. pylori* to be either strongly pathogenic or non-pathogenic.

To summarise, virulence factors of both Iraqi and Iranian *H. pylori* strains appear more closely related to Western countries than to Asian countries. Iranian strains appear more virulent, but the difference appears unlikely completely to explain the difference in disease prevalence between these countries. This suggests that unidentified strain, host and environmental factors may contribute to these differences. In the absence of East Asian type *cagA* and almost universally virulent strains (as found in Japan and parts of China) the very high gastric cancer rate in Iran remains largely unexplained. Similarly, the cancer rates in Iraq appear lower than would be expected from the circulating *H. pylori* strain types - an enigma similar to that reported (controversially) in Africa (Holcombe, 1992).

4. The Gastric Mucosal Cytokines Response in Kurdish Iraqi Patients, and Particularly T helper Differentiation.

Abstract

Introduction: *H. pylori* induces adaptive immunity, but the balance of the T-helper (Th) response varies between individuals; a more pro-inflammatory T-helper 1(Th1) response has been associated with the development of gastric adenocarcinoma. In Kurdistan region, Iraq, the incidence of gastric cancer is unusually low. Thus we now studied the T-helper response in patients from Iraq by measuring the Th subset-associated cytokines in gastric biopsy samples and IgG1/ IgG2 responses. We also investigated associations of smoking and gender with Th responses because being a smoker is linked to an increased risk of peptic ulcer and gastric cancer, and gastric cancer is more common in men. We have checked these associations in an independent population from the UK.

Materials and Methods: Gastric biopsies were collected from 44 (35 *H. pylori*-infected) and 27 (14 *H. pylori*-infected) Kurdish Iraqi and UK patients, respectively. Real-time PCR was performed to quantify the mRNA levels of *IFNγ* and *interleukin-12 p35* (*IL-12*) (typically Th1 cytokines), *IL-4* and *IL-10* (Th2 cytokines), and *FOXP3* (Treg response). Serum IgG subclasses were measured by ELISA to confirm Th subset data.

Results: For Iraqi samples, the levels of *IFNγ*, *IL-12 p35, IL-10, IL-4* and *FOXP3* mRNA was found to be elevated in gastric mucosal samples from *H. pylori*-infected patients compared to those from *H. pylori*-negative patients (median increase 7-fold p=0.001; 17-fold p=0.002; 1320-fold p=0.001; 1184-fold p=0.001; and 3-fold p=0.01, respectively, confirming a predominant IL-4 and IL-10 (Th2) response). Interestingly, *IFNγ* mRNA levels (Th1) were 16-fold higher in tissues taken from 17 smokers than found in tissues taken from 18 non-smokers (p=0.009), and *IL-4* mRNA levels (Th2) found in tissues taken from 20 females were 40-fold higher than found in tissues taken from 15 males (p=0.005). To confirm our results, we studied serum IgG subclass responses to *H. pylori*. The results showed that there was a 4-fold decrease in the *H. pylori*-specific IgG1:IgG2 levels in smokers compared to non-smokers (p=0.0013), consistent with a biased Th1 response amongst smokers, although no associations were found with gender. To further address whether smoking enhances a Th1 response, the effect of smoking on gastric

IFNγ mRNA levels was studied in an independent population from the UK. Levels of *IFNγ* mRNA were 6-fold higher in gastric biopsies from 5 smokers than from 9 non-smokers (p=0.02).

Conclusion: The predominant Th2 response to *H. pylori* in Kurdistan, Iraq may contribute to the low cancer incidence. The association between a Th1 response to *H. pylori* and smoking suggests that smoking may promote a pro-inflammatory Th1 response and we speculate that this may help explain the association between smoking and gastric cancer.

4.1 Introduction

Helicobacter pylori infection causes peptic ulceration and gastric adenocarcinoma. In Iraq, despite the early acquisition of *H. pylori* infection (Hussein et al., 2008b), gastric cancer is rare (GLOBOCAN, 2002). Three factors interact to determine the clinical outcome of *H. pylori* infection; the virulence of the infecting *H. pylori* strain, the type and extent of the host immune response to infection, and modulating cofactors such as smoking, gender and diet (Atherton, 2006). Virulence factors of Iraqi *H. pylori* strains appear more closely related to Western countries and unlikely to explain the low cancer rate completely (Hussein et al., 2008c).

H. pylori strains can induce both arms of adaptive immunity: local and systemic antibody production and cell-mediated responses (Atherton, 2006). T-helper 1 (Th1), Th2 and regulatory T (Treg) cells are activated by *H. pylori* (Robinson et al., 2008, Robinson et al., 2005). Studies in animal models and supportive data in humans show that a strong Th1 response increases the severity of gastritis and the risk of gastric cancer (Akhiani et al., 2002, Bamford et al., 1998, Blaser, 1992). In contrast, a Th-2 response is associated with mild gastritis and a lower risk of gastric cancer (Atherton, 2006, Robinson et al., 2005). Observational data from humans show that gastric T cells in gastric ulcer patients are more polarized to Th1 than in duodenal ulcer patients, suggesting that a distinct immune response to *H. pylori* may be associated with different disease outcomes (Itoh et al., 2005).

Regulatory T-cells (Tregs) are thought to be important in suppressing deleterious immune and inflammatory responses (Bamford et al., 1998, Mohammadi et al., 1996). Tregs are reduced in patients with peptic ulceration. *FOXP3* is a marker for

Tregs: it encodes a transcriptional repressor and acts as a master regulator in their development and function (Hori et al., 2003, Robinson et al., 2008). Th-1 cells, whose differentiation is triggered by interleukin-12 (IL-12) secretion, predominantly secrete interferon gamma (IFNγ) and IL-2, while Th-2 cells, whose differentiation is stimulated by IL-10 secretion, secrete IL-4, IL-5, IL-9, and IL-13 (Atherton, 2006, Bergman et al., 2004, Robinson et al., 2008, Smythies et al., 2000). 90% of *H. pylori*-infected individuals develop *H. pylori*-specific IgG antibodies particularly IgG1 and IgG2 (Blaser, 1992, Bontkes et al., 1992). While Th1 responses are associated with reduced IgG1 and/or increased IgG2, the association of Th2 with IgG responses is weak in human (Mitchell et al., 2001, Vorobjova et al., 2006).

As well as determining the nature of the Th cytokine responses in gastric biopsies from Kurdish patients, we were interested in the potential drivers of that response. The main potential drivers that we were able to study were bacterial virulence factors (in particular the major virulence factors CagA and VacA) and environmental factors (particularly smoking and gender (Atherton, 2006, Stoicov et al., 2004, Walker and Taylor, 1979)). Little is known about the relationship between virulence factors and Th differentiation. In a recent study in vivo in humans we showed no association between *H. pylori* virulence factors and specific Th responses (Robinson et al., 2008). However, in vitro studies have shown that VacA can supress T cell proliferation (Schmees et al., 2006, Sundrud et al., 2004).

Smoking is a risk factor for a wide range of diseases including lung and prostate cancer (Batty et al., 2008). In addition, it has been shown that smokers are more

prone to peptic ulceration and gastric cancer than non-smokers (Ko and Cho, 2000, Maity et al., 2003, Sonnenberg, 1988, Walker and Taylor, 1979). One possibility is that, smoking is associated with these diseases because it modulates the immune system. In an *in vitro,* study it was shown that tobacco-exposed lymphocytes have reduced proliferative capacity (Barbour et al., 1997). In a study conducted using lung biopsies, smoking was associated with increased expression of *IFNγ* (Di Stefano et al., 2004). Thus we hypothesised that smoking might be associated with Th1 responses.

As sex hormones differ between genders, many researchers have studied the effect of sex hormones on the immune response. It has been shown that women produce a stronger Th2 cellular and humoral immune response and suffer from a higher incidence of Th2-associated autoimmune diseases e.g. multiple sclerosis (Ansar et al., 1985). Faas et al. argued that the production of IL-4 is significantly higher in the luteal phase of the menstrual cycle as compared with the follicular phase (Faas et al., 2000). On the other hand, it is agreed that T lymphocyte counts are lower in males than in females. This may be due to increased testosterone concentrations, as testosterone is associated with apoptosis in T cells (McMurray et al., 2001). Hence, we hypothesised that female gender might be associated with Th2 responses to *H. pylori* in the stomach.

Thus we studied Th1/Th2 responses in *H. pylori*-infected subjects in Kurdistan region in northern Iraq and factors that might affect such responses. We found that smoking was associated with a Th1 response. Because of the importance of this we

confirmed our results by another methodology (IgG subclass ELISA) and in a different (UK) population.

4.2 Materials and Methods

4.2.1 Patients and Serum Samples

Gastric biopsies were obtained from 44 Kurdish Iraqi patients (35 were *H. pylori*+: duodenal ulcer (DU) 11; gastric ulcer (GU) 5; no ulcer (NUD) 19, all non-infected were NUD) and 27 UK patients (14 *H. pylori*+: GU 3; DU 3 and NUD 8, all non-infected were NUD) undergoing routine upper GI endoscopy to investigate dyspepsia with approval from Hospitals Ethics Committees. Mean age ± standard deviation of the patients from Iraq was 37±18 years and from the UK was 56±11. During gastroscopy, 2 biopsy samples were taken from the antrum and either placed in 1 ml of isosensitest broth (Oxoid, Basingstoke, UK) containing 15% (v/v) glycerol and stored in liquid nitrogen or placed in RNAlater solution and preserved for mRNA analysis. DNA was extracted directly from the biopsy specimens and used for PCR-based *H. pylori* typing. At the time of endoscopic examination 15 ml of blood was drawn from each Iraqi patient. Serum was separated and samples were stored at -20 °C until use in the IgG subclass enzyme linked immunosorbent assay (ELISA).

4.2.2 cDNA Synthesis

Total RNA was extracted and purified from gastric biopsies using the total RNA Isolation System (Qiagen, Crawley, UK). RNA in the samples was quantified using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies Wilmington, DE, USA). The synthesis of cDNA from 50 ng RNA was conducted by using SuperScript II reverse transcriptase (Invitrogen) with oligo(dT) primer (Invitrogen) as per the manufacturer's instructions.

4.2.3 Real Time PCR

RT-PCR was performed on a Rotor-Gene 3000 (Corbett Research, Mortlake, Sydney, Australia) by using the DyNAmo HS SYBR green qPCR kit (GRI, Braintree, Essex, UK). A reaction volume of 20 µl was used. No-template controls were included in each cycle and a melting curve analysis was performed to ensure the specificity of primers (Figure 4.1). To ensure that products were not amplified from contaminated genomic DNA, first stage RT-PCR samples produced in the absence of reverse transcriptase from each RNA template were tested in parallel. Samples were run in duplicate and results were analysed according to the method described by Pffafl (Pfaffl, 2001). Relative expression levels of the gene of interest *IL-4, IL-10, IFNγ, FOXP3* (Lundgren A et al., 2003, Robinson et al., 2008) and *IL-12 p35* were determined by normalising against a housekeeping gene (*GAPDH*) (Walker et al., 2003), and then comparing this value to the normalised level in cDNA made from a pooled RNA preparation from 5 *H. pylori*-negative patients to obtain a fold difference. A commercial pooled human cDNA standard (BD Biosciences, San Diego, CA) was included as a positive control in all assays. Amplification of *IL-4, IL-10, IFNγ* and *FOXP3* was carried out over 45 cycles of 15 seconds at 95^{o}C, 30 seconds at 61^{o}C and 30 seconds at 72^{o}C. IL-12 amplification was carried out over 45 cycles of 15 seconds at 95^{o}C, 30 seconds at 62^{o}C and 30 seconds at 72^{o}C. Commercial primers were used for human IFNγ (SuperArray RT, Tebu-Bio, Peterborough, UK). Other PCR primer sequences are shown in Table 4.1.

Table 4. 1 Primer sequences and efficiencies for real-time PCR

Gene	Forward primer 5'-3'	Reverse primer 5'-3'	Efficiency
GAPDH(Walker et al., 2003)	CCACATCGCTCAGACACCAT	GGCAACAATATCCACTTTACCAGAGT	1.72
FOXP3 (Lundgren A et al., 2003)	CAGCACATTCCCAGAGTTCCT	GCGTGTGAACCAGTGGTAGAT	2.07
IL-4 (Robinson et al., 2008)	GCCCTGCAGAAGGTTTCCTT	AACAGCCTCACAGAGCAGAAGAC	1.73
IL-10	GCTGGAGGACTTTAAGGGTTACCT	CTTGATGTCTGGGTCTTGGTTCT	1.94
IL-12 p35	CTGAGGAGAGTCTGCCCATTG	TGGGTGGGTCAGGTTTGATG	3.18

A

$$\text{Ratio} = \frac{(E_{target})^{\Delta CP target\ (control - sample)}}{(E_{ref})^{\Delta CP ref\ (control - sample)}}$$

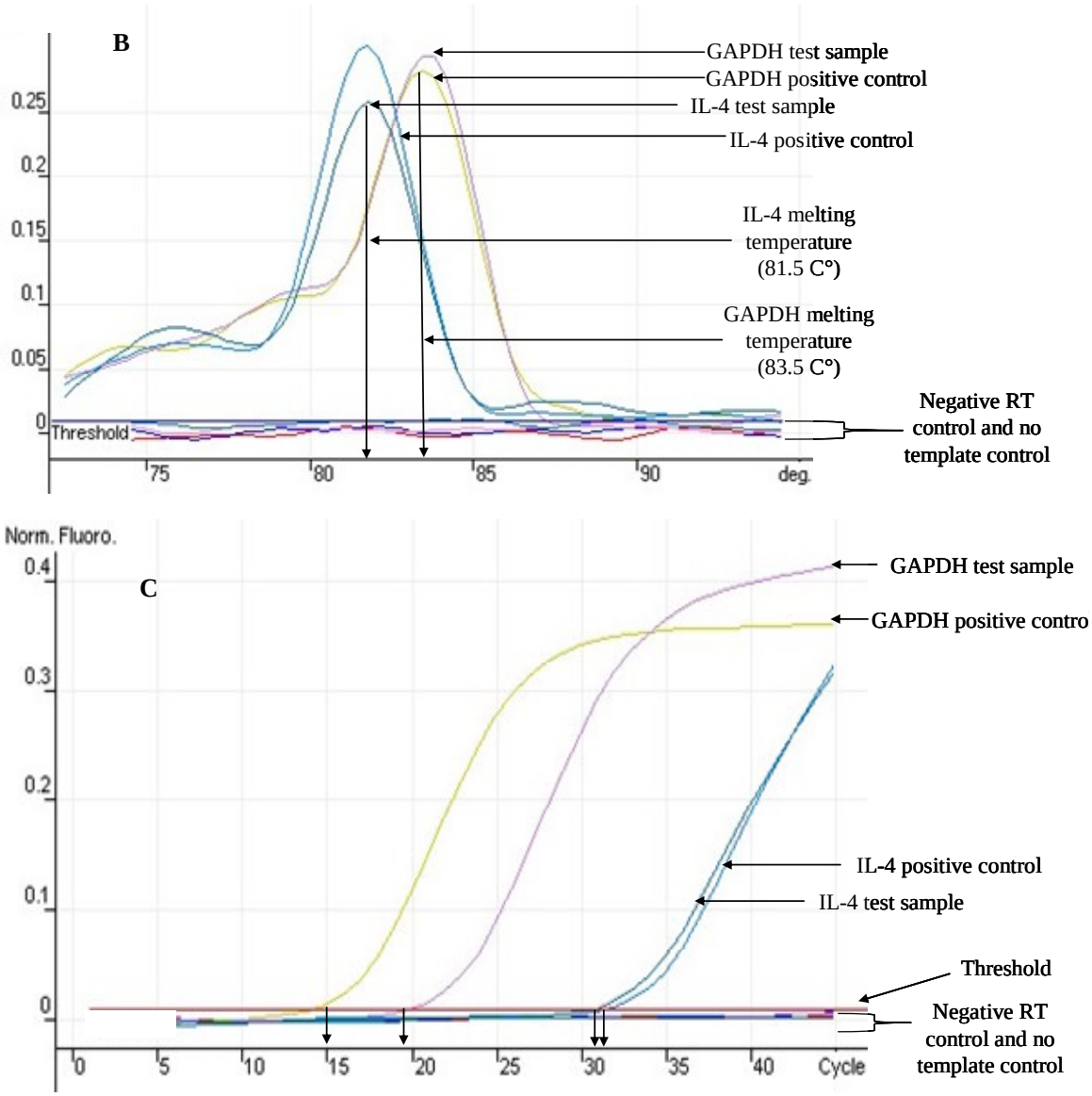

Figure 4. 1 Pffafl equation and examples of melt and amplification curves produced by IL-4 RT-PCR rotor.

This plot shows the intensity of fluorescence at different temperatures. Product of the same size show a fluorescence peak at the same temperature. (A) Pffafl equation (B) melting temperature for IL-4, (C) amplification for IL-4. Small arrows indicate CT values (Cycle number at which the set threshold crosses). Norm. Fluoro: Normalised Fluorescence; deg: C° degree.

4.2.4 Efficiency Calculation

To calculate relative expression levels of the gene of interest relative to the level of housekeeping gene transcript using the Pfaffl method (Pfaffl, 2001), the reaction efficiency of each PCR was calculated (Table 4.1). The PCR efficiencies were calculated by serially diluting the positive control cDNA (BD Biosciences, Oxford, UK) and calculating the gradient from plots of dilution against CT value, which is the cycle number at which the threshold was crossed. Examples of plots calculated are shown in figure 4.2.

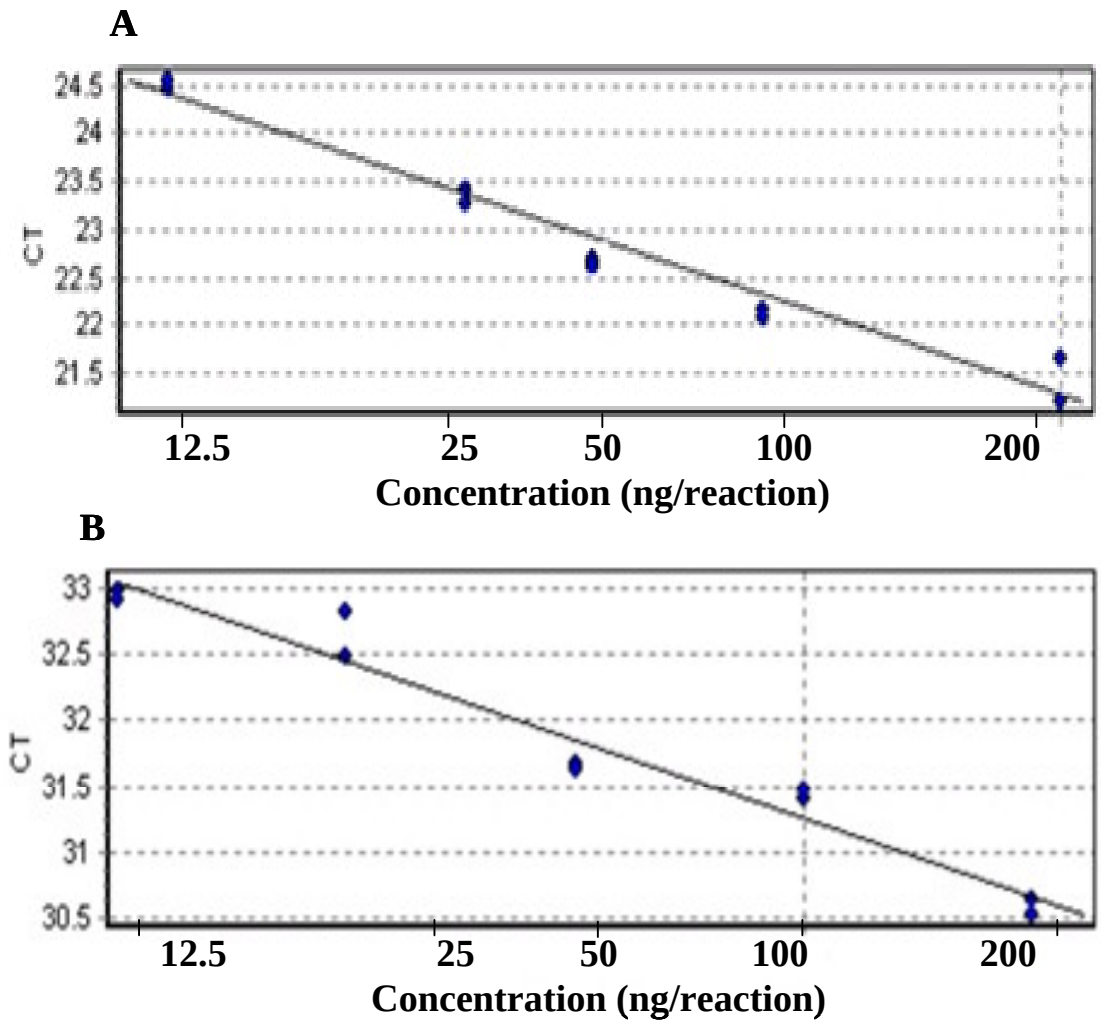

Figure 4. 2 Efficiency runs for IL-10 (A) and IL-12 (B) PCRs.

The PCR efficiencies were calculated by serially diluting the positive control cDNA (BD Biosciences, Oxford, UK) and calculating the gradient from plots of dilution against CT value, which is the cycle number at which the threshold was crossed. The efficiency of IL-10 reaction was 1.94 (R^2=0.98) and the efficiency of IL-12 reaction was 3.2 (R^2=0.96).

4.2.5 Antigen Preparation for ELISA

A lysate of 5 Iraqi stains (all of which are *cagA*-positive) was prepared. The optical density of 5 separate bacterial suspensions was equalized and samples were then pooled together. Then, the lysate was prepared by sonicating a suspension of a pool of the 5 Iraqi strains in PBS. This procedure was performed on ice at 10 amplitude microns in six cycles of 30 seconds, followed by 30 seconds cooling. The protein concentration was determined using a modified Lowry protein assay kit (Bio-Rad) and BSA as a standard curve.

4.2.6 Serum IgG Subclass ELISA

A multiple serum dilution series ELISA method based on that of Robinson et al (Robinson et al., 1997) was used to quantify anti-*H. pylori* IgG. Wells of a 96 well plate (NUNC maxisorp, Roskilde, Denmark) were coated with 50 µl *H. pylori* sonicate antigen diluted to 10µg/ml in 0.1M carbonate/bicarbonate coating buffer (pH 9.6). The plates were then incubated overnight at 4°C. After washing with PBS-Tween (PBS with 0.05% Tween 20), 100 µl of blocking solution (3% BSA in PBS-Tween) was added to each well and the plates were incubated for one hour at room temperature. After washing as previously described, 50 µl serum samples were added in duplicate in a doubling dilution series of up to 1/12800. The diluent used was 1% BSA in PBS-Tween. Plates were then incubated for 90 minutes at room temperature then washed three times followed by two one minute soaks in PBS-Tween. 50µl per well of diluted (1/3,000) anti-human IgG1-biotin conjugate (Sigma Aldrich, St. Louis, MO, USA) in PBS/Tween or diluted (1/100000) anti-human IgG2-biotin conjugate (Sigma Aldrich, St. Louis, MO, USA) was added to the wells and they were incubated for a further 90 minutes. The plates were then

washed three times followed by two one minute soaks in PBS-Tween then 50 µl diluted streptavidin peroxidise (1/1000) (Sigma Aldrich, St. Louis, MO, USA) was added and the plates were incubated for 30 minutes. The plates were washed three times followed by two one minute soaks in PBS-Tween then 100 µl of Tetra methylbenzidene substrate (eBioscience) was added to each well. After incubation for 15 min, the reaction was stopped by adding 50µl of 2M H_2SO_4. The optical density of the samples was measured using a microplate reader (Labsystems iEMS Reader MF, Helsinki, Finland) at a wavelength of 450nm with a reference wavelength of 650nm. Then, curves of dilution against mean OD for each sample were plotted.

A pool of negative control sera from 4 uninfected donors was applied to 5 replicate wells at a 1/50 dilution on each plate. The average of these wells was used as a cut off point to calculate the end point titre for each sample. No serum, no secondary antibody or no streptavidin horseradish peroxidase were included as assay controls, where diluent was used instead.

4.2.7 Statistics

Data were analyzed using the Minitab 15 software program. Boxes represent the range of the first and third quartiles, the median values are shown as horizontal lines within the bars and the whiskers depict the lowest and highest observations within 1.5 times the first to third inter-quartiles ranges. Statistical tests of unpaired data were carried out using the the Mann-Whitney U-test. A significant difference was taken at $p \leq 0.05$ unless otherwise stated.

4.3 Results

4.3.1 Gastric Immune Response in Kurdish Iraqi Samples

H. pylori can induce Th1, Th2 and regulatory T cell responses (Robinson et al., 2008). We investigated the likely responses in the stomachs of Iraqi patients by performing real-time PCR on mucosal biopsy specimens. In infected compared to uninfected biopsies, there were significantly increased levels of IFNγ, a Th1 cytokine, (median± standard error, *H. pylori*+: 8.6±63; *H. pylori*-: 1.2±0.1, median increase 7-fold, p=0.001) (Figure 4.3A). To confirm the Th1 biased responses, levels of *IL-12p35* mRNA, were studied in *H. pylori*-infected and non-infected samples. A 17-fold increase was observed in *H. pylori*-infected specimens (*H. pylori*+: 30.9±9; *H. pylori*-: 1.8±0.3 p=0.002) (Figure 4.3B). Next to assess whether there was a higher or a lower Th2 response, we measured levels of *IL-4* mRNA, a Th2 cytokine in infected and uninfected biopsies. Levels of *IL-4* mRNA were also consistently higher in *H. pylori*-infected patients than uninfected patients, but differences were more marked (*H. pylori*+: 153±177; *H. pylori*-: 0.26±0.1, 1320-fold, p=0.001) (Figure 4.3C). To confirm the Th2 biased responses, *IL-10* mRNA levels, which may also be associated with a Th2 response, were measured. A 1184-fold elevation in *IL-10* mRNA level was found in *H. pylori*-positive samples (*H. pylori*+: 54±39; *H. pylori*-: 0.04±0.28 p=0.001) (Figure 4.3D). Individual specimen samples showed a mixed Th subclass response. However, in most specimens a well-polarized Th response was found (Figure 4.4).

Because IL-10 is secreted by Tregs as well as Th2 cells, we examined a separate Treg marker (*FOXP3* mRNA levels) in gastric mucosa. The levels of *FOXP3* mRNA were found to be elevated in samples from *H. pylori*-infected patients

compared to those from *H. pylori*-negative patients but only by 3-fold (*H. pylori*+: 3.7±1.7; *H. pylori*-: 1±0.1 p=0.01) (Figure 4.3E). This suggests that the main source of IL-10 in gastric mucosa may be Th2 cells.

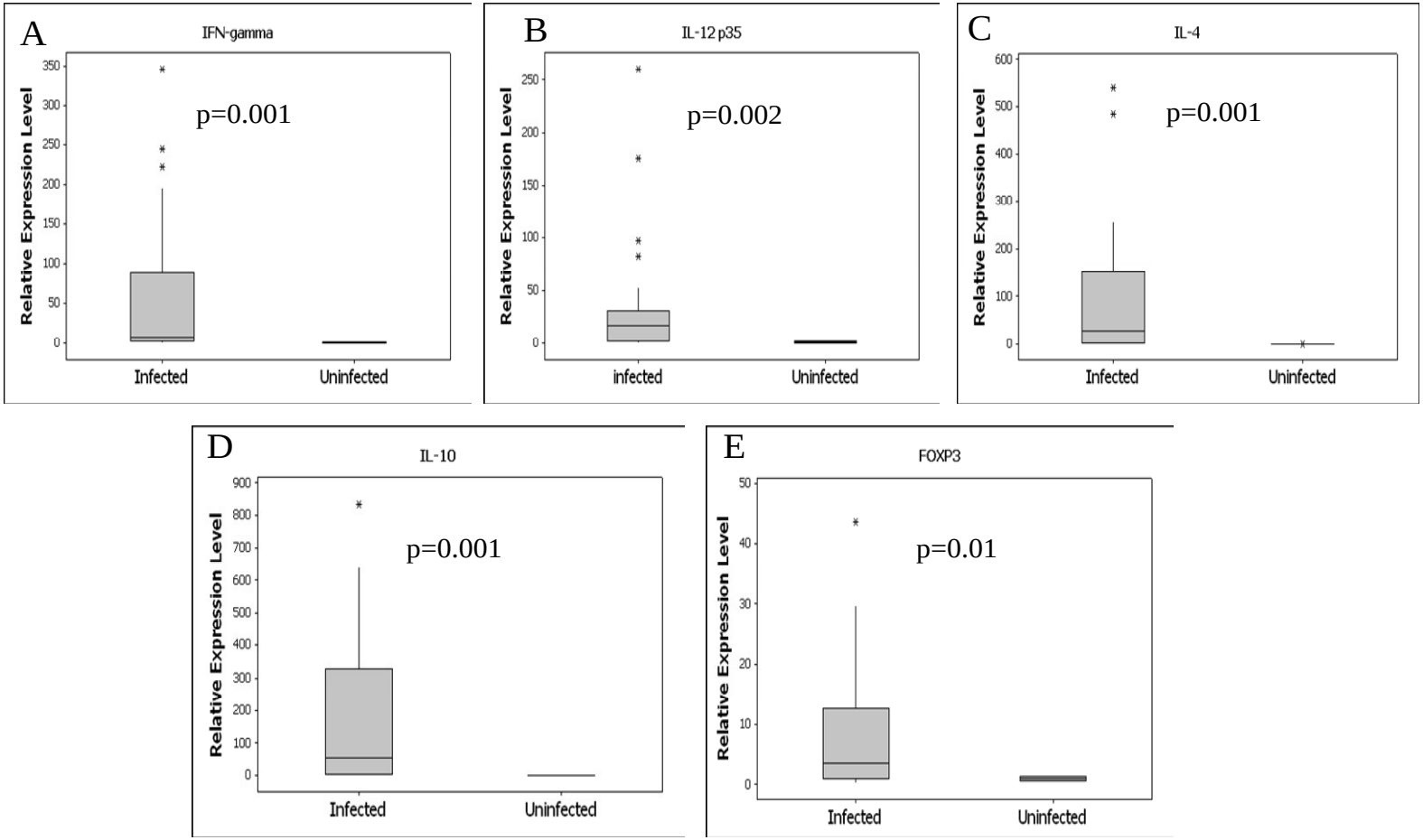

Figure 4. 3 Relative *IFNγ*, *IL-12p35*, *IL-4*, *IL-10* and *FOXP3* mRNA levels in gastric biopsies from 35 *H. pylori*-positive Kurdish Iraqi and 9 *H. pylori*-negative subjects. * Outlier value.

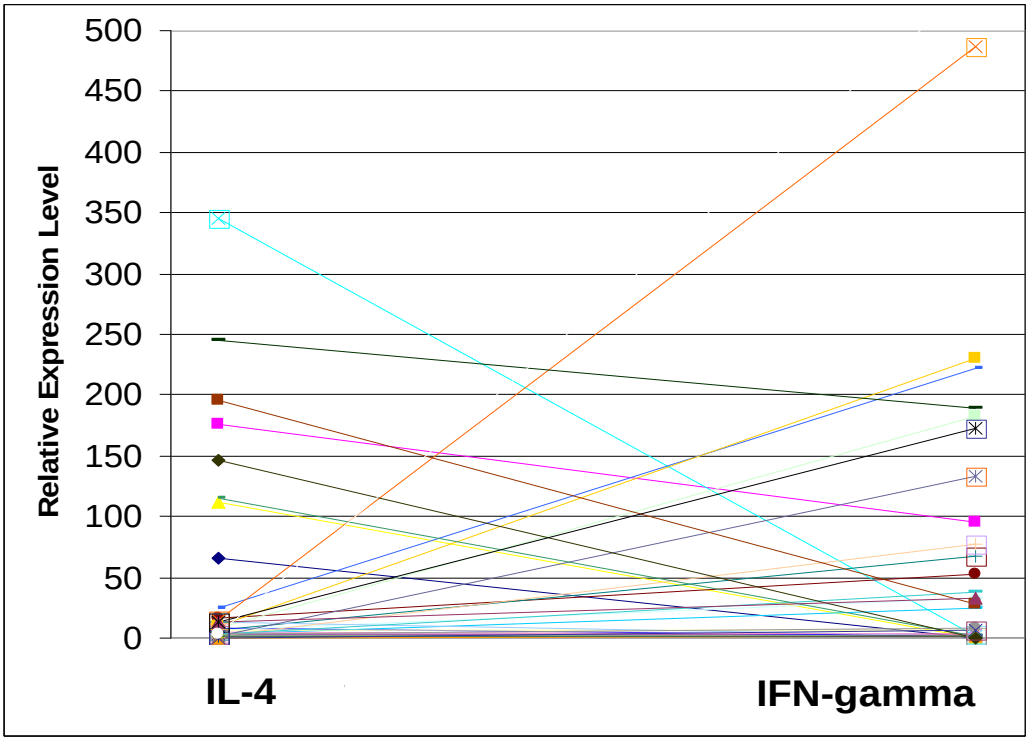

Figure 4. 4 *IL-4* and *IFNγ* mRNA level in gastric biopsies taken from 35 *H. pylori*-positive Kurdish Iraqi patients. Lines represent the results obtained from individual biopsies. Some patients showed extremely polarised T-helper responses.

4.3.2 Association between Gastric Cytokines and Clinical Outcome

To test whether expression levels of specific cytokines correlated with the development of GU, DU, and NUD, data are re-stratified according to these clinical outcomes. No significant associations were found between disease status and expression of any of the genes we studied. However, *IFNγ* mRNA was numerically increased by 10-fold amongst the DU samples (median DU: 65; GU: 15; NUD: 6 p=0.19).

4.3.3 Relationship between Gastric Cytokines and *H. pylori* Virulence Factors

Having shown that there is a well-polarised immune response in most of the samples, we next examined whether the immune responses were associated with bacterial virulence factors. Thus we re-stratified our data according to *cagA* and *vacA* status. The levels of *IFNγ* mRNA were 2-fold higher in tissues infected with *cagA*+ strains compared to *cagA*- samples but the difference was not significant (median *cagA*+: 12.6; *cagA*- : 6.3 p=ns). In addition, the levels of *IL-12* mRNA were 5-fold higher in tissues infected with *cagA*+ strains compared with *cagA*- samples, but still this was not significant (*cagA*+: 20.8; *cagA*- : 3.9 p=ns) (Figure 4.5). No differences were found in the levels of *IL-4, IL-10* and *FOXP3* mRNA between tissues infected with *cagA*+ and *cagA*- *H. pylori*. No significant associations were found between *vacA* signal, mid and intermediate region type mucosal levels of any of the cytokines mRNAs studied (Figure 4.6).

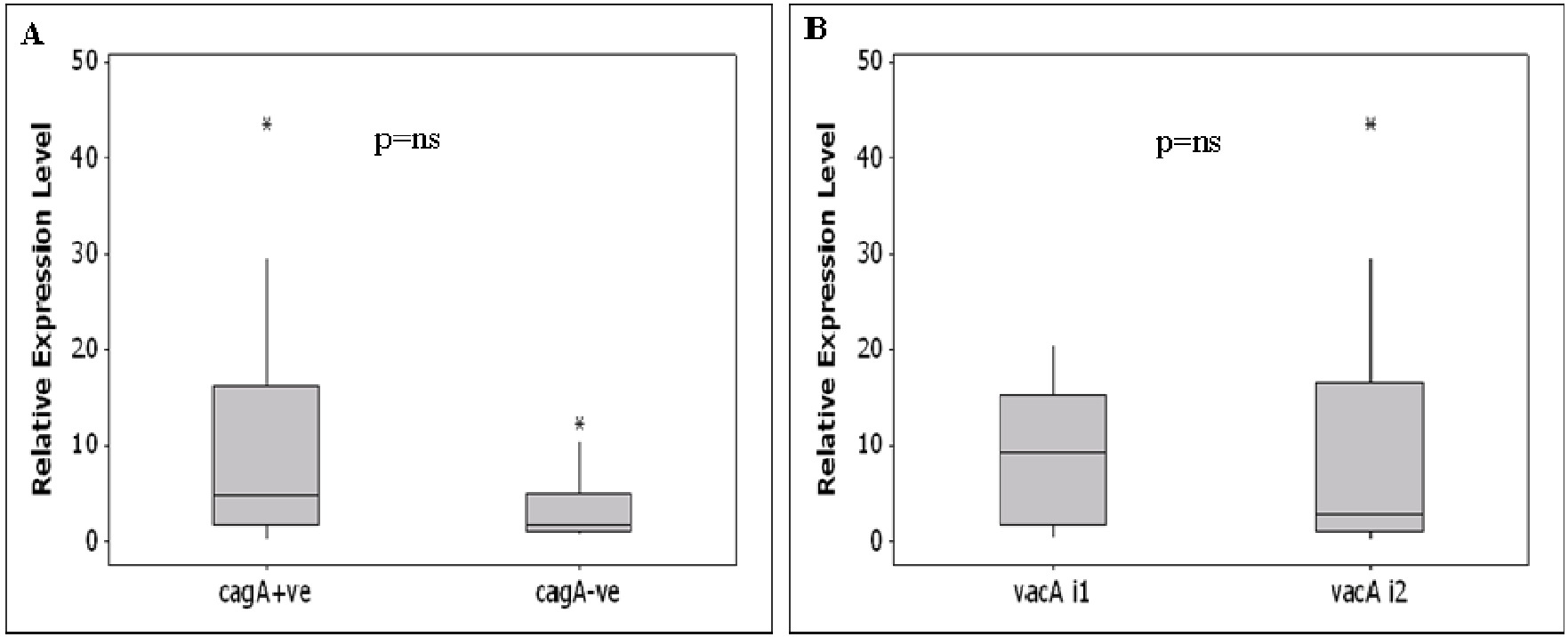

Figure 4. 5 Relative *FOXP3* mRNA levels in gastric biopsies from *H. pylori*-infected Kurdish Iraqi patients in relation to different virulence factors. Real-time quantitative PCR analysis of *FOXP3* gene expression relative to GAPDH expression in 35 *H. pylori*-infected patients. No significant difference (NS) was found between *cagA*-positive versus *cagA*-negative infected patients (A), or *vacA* i1 versus *vacA* i2 infected patients (B). * Outlier.

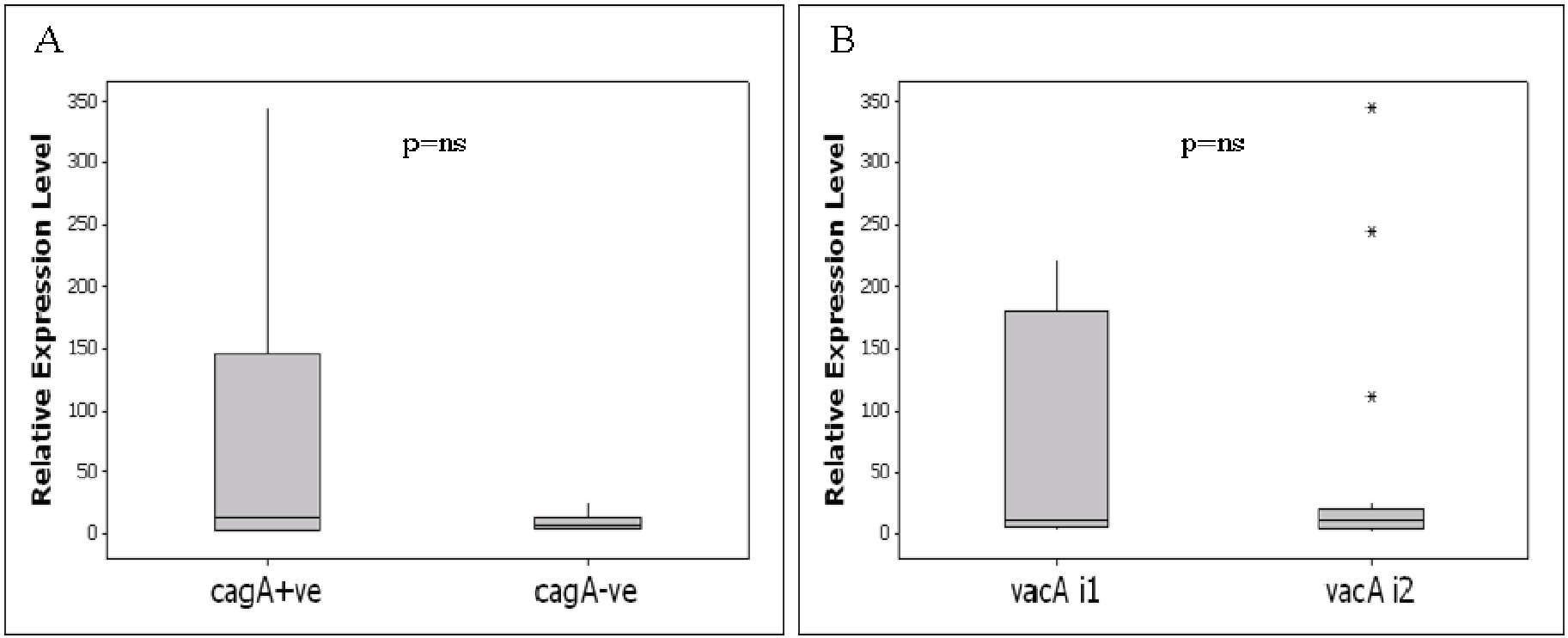

Figure 4. 6 Relative *IFNγ* mRNA levels in gastric biopsies from *H. pylori*-infected Kurdish Iraqi patients in relation to different virulence factors. Real-time quantitative PCR analysis of *IFNγ* gene expression relative to GAPDH expression in 35 *H. pylori*-infected patients. No significant difference (NS) was found between *cagA*-positive versus *cagA*-negative infected patients (A) or *vacA* i1 versus *vacA* i2 infected patients (B). * Outlier**.**

4.3.4 Effect of Smoking and Gender on Gastric Cytokines

As virulence factors could not explain the bias in immune responses, we decided to study other factors that might affect the immune responses: smoking and gender. *IFNγ* mRNA levels were 16-fold higher in tissues taken from 17 smokers than found in tissues taken from 18 non-smokers (smokers: 111±524; non-smokers: 5.3±5.8 p=0.01) (Figure 4.7A). Levels of *IL-12* mRNA were 6.6-fold higher in smokers than non-smokers (p=0.07) (Figure 4.8). These results suggest a correlation between Th1 responses and smoking.

Next we assessed whether Th1 cytokine was associated with male sex. We found no association for *IFNγ* or for IL-12. However, *IL-4* mRNA levels found in tissues taken from 20 females were 40-fold higher than *IL-4* mRNA levels found in tissues taken from 15 males (p=0.005) (Figure 4.9A). Additionally, we found the levels of *IL-10* mRNA were 89-fold higher in female than male (p=0.01) (Figure 4.10). *FOXP3* mRNA was not found to be associated with either smoking or gender.

4.3.5 IgG subsets ELISA

To confirm the correlation between smoking and gender and the immune response using another technique, serum IgG subclass responses to *H. pylori* were examined. Th1 responses are associated with reduced IgG1 and increased IgG2 (Mitchell et al., 2001). We found a 4-fold decrease in the *H. pylori*-specific IgG1:IgG2 ratio in smokers compared to non-smokers (smokers: 0.3±0.2; non-smokers: 1.4±0.5 p=0.0013) confirming a predominant Th1 responses (Figure 4.11). No relationship between gender and IgG subclass could be found. To explore these results the relationship between IgG subclass and gastric cytokines was studied. As expected, a significant inverse relationship was found between IgG1:IgG2 ratio and *IFNγ* mRNA levels (p=0.047 Rs=-0.34). No correlation between IgG subclass and Th2 cytokines could be established.

4.3.6 Effect of Smoking and Gender in the UK

Finally, we addressed whether our results were confined to our Iraqi population or found more generally. We had a small amount of cDNA available from gastric biopsies of patients from the UK. Thus we decided to do RT-PCR for *IFNγ* and *IL-4* mRNA. In agreement with the Iraqi data, we found that *IFNγ* mRNA levels were 6-fold higher in tissues taken from 5 smokers than found in tissues taken from 9 non-smokers (p=0.02) (Figure 3B). *IL-4* mRNA levels were similar between these groups. With regard to gender, no differences in *IFNγ* or *IL-4* mRNA levels were found between 7 males and 7 females (Figure 4B).

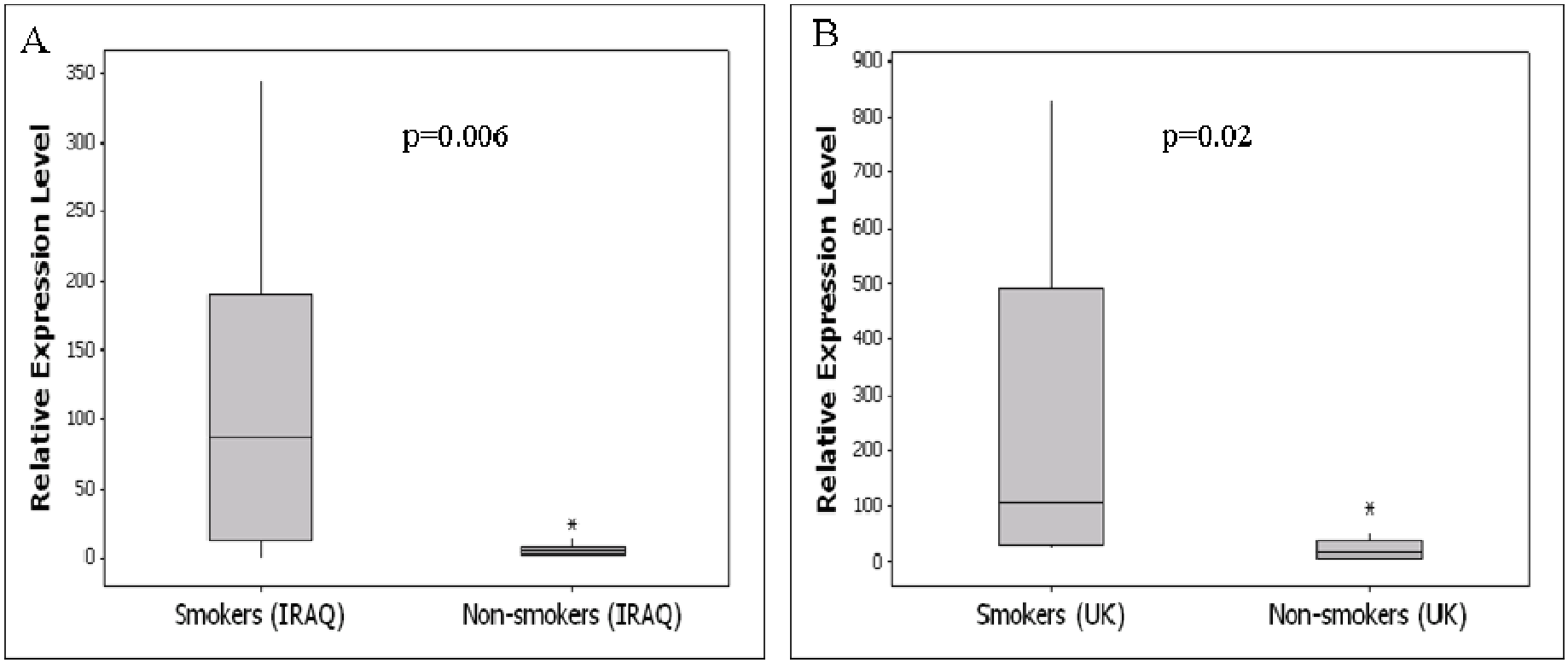

Figure 4. 7 Relative *IFNγ* mRNA levels in gastric biopsies from 17 *H. pylori*-infected smokers and 18 non-smokers in Kurdistan region, Iraq and 5 smokers and 9 non-smokers in the UK. * Outlier value.

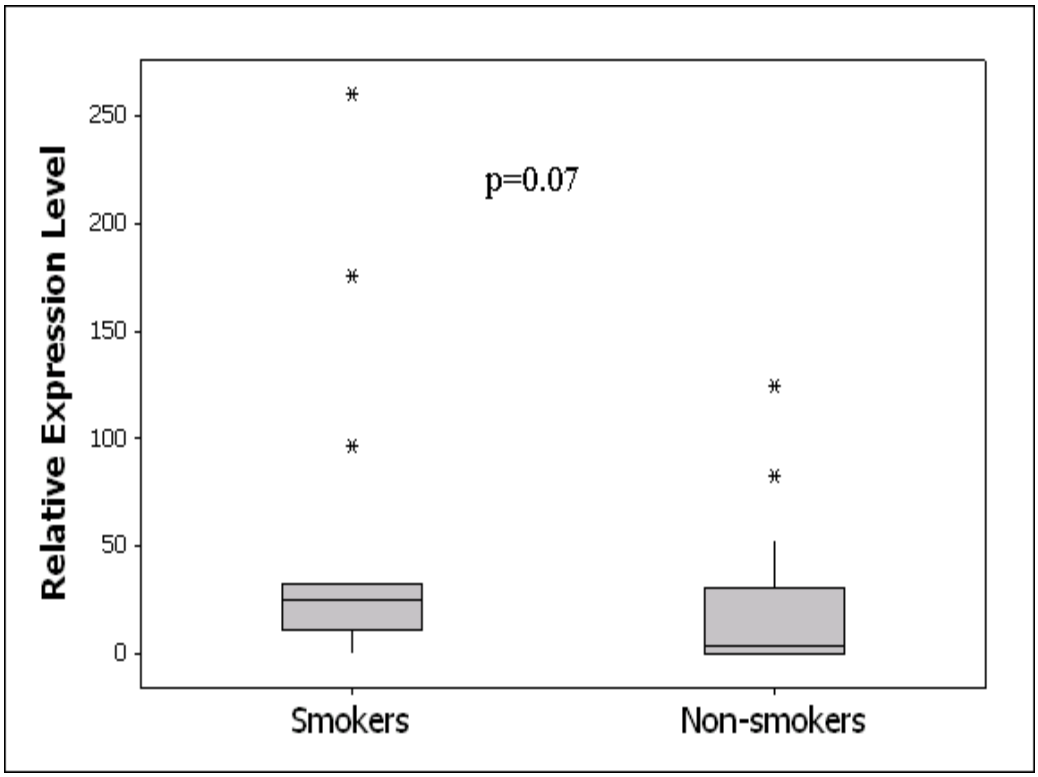

Figure 4. 8 *IL-12p35* mRNA level in gastric biopsies taken from Kurdish Iraqi patients, 17 smokers and 18 non-smokers. * Outlier.

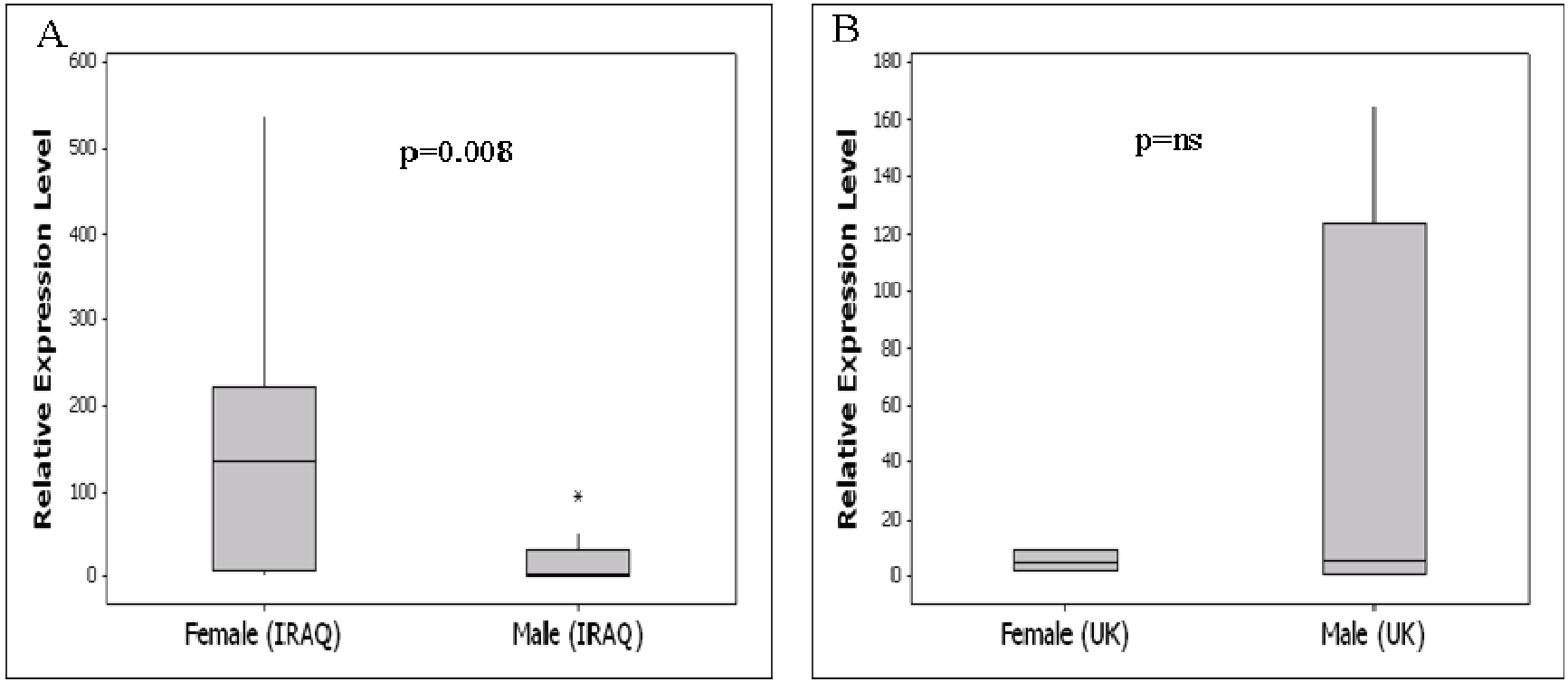

Figure 4. 9 Relative *IL-4* mRNA levels in gastric biopsies from 15 *H. pylori*-infected males and 20 females in Kurdistan region, Iraq and 7 males and 7 females in the UK patients. * Outlier. ns= no significant difference.

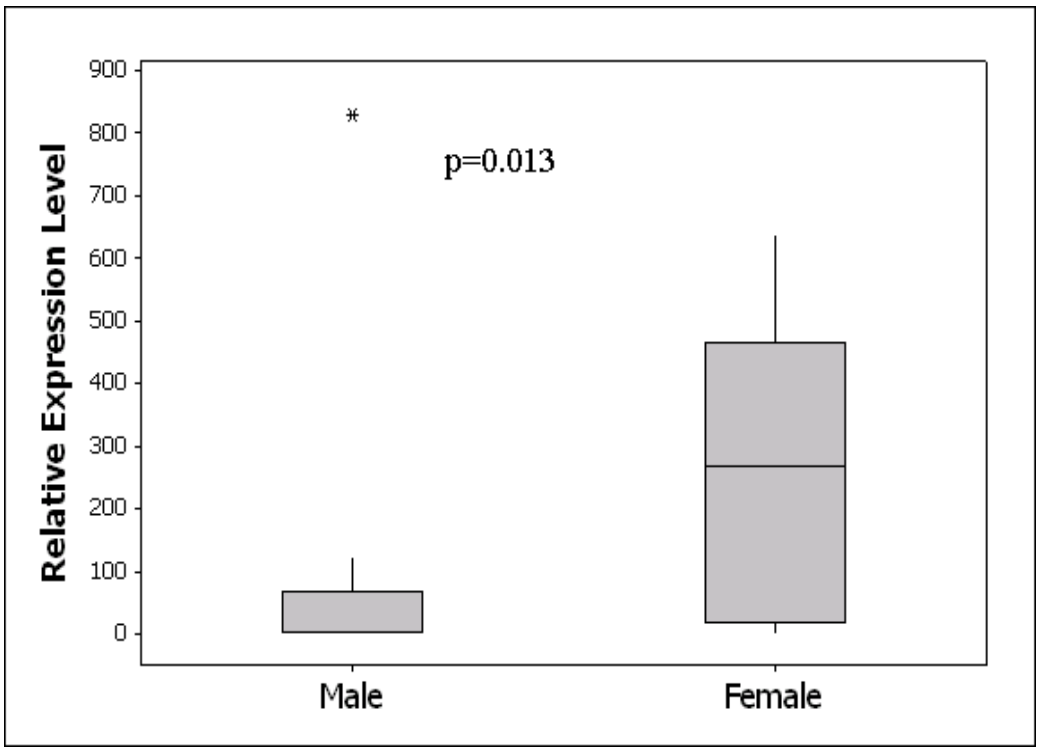

Figure 4. 10 *IL-10* mRNA level in gastric biopsies taken from Iraqi patients, 20 females and 15 males. * Outlier.

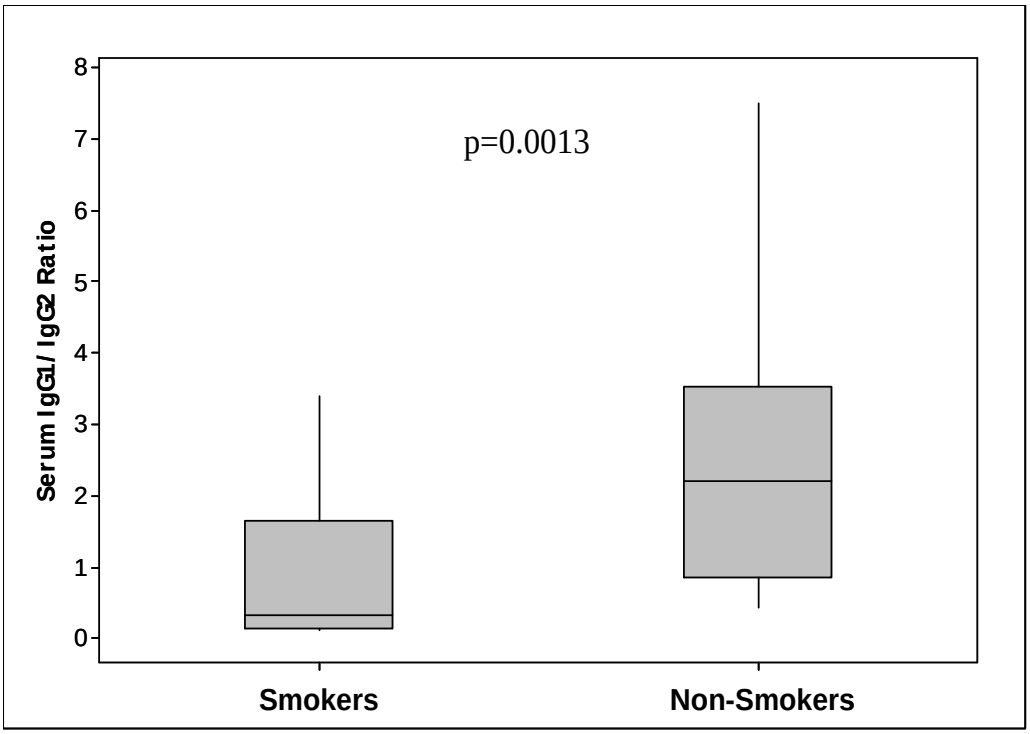

Figure 4. 11 Anti-*H. pylori* serum IgG1 and IgG2 responses of 17 smokers and 18 non-smokers amongst the group of infected Iraqi patients.

4.4 Discussion

In this study, the levels of *FOXP3, IFNγ, IL-12, IL-4* and *IL-10* expression were significantly higher in infected than uninfected Kurdish Iraqi patients. This agrees with Robinson et al. who found a mixed T-helper immune response against *H. pylori* (Robinson et al., 2008), and D'Elios et al. (D'Elios et al., 2003). Surprisingly, the increase in Th1 and Th2 cytokines was not similar. We found a 7-fold increase in *IFNγ* and 17-fold increase in *IL-12p35* mRNA levels compared to 1320-fold increase in *IL-4* and 1184-fold increased *IL-10* mRNA levels suggesting a predominant Th2 responses. As *FOXP3* mRNA levels increased by 3-fold only, the IL-10 is likely to be secreted mainly from Th2 cells rather than Tregs. A study conducted in the UK suggested a more balanced Th1/Th2 responses in this country: in infected compared to uninfected tissues, there were significantly increased levels of *IFNγ* (119-fold) and *IL-4* (231-fold) mRNA (Robinson et al., 2008). This provides some evidence that Th2 cell responses might be the main responses to *H. pylori* infection in Kurdistan region, Iraq. This agrees with a study conducted in Africa where Th2 responses were found to predominate. In Africa, like Iraq, gastric cancer is thought to be rare (Mitchell et al., 2002).

Several studies have addressed the role of the balance between induction of proinflammatory cytokines and stimulation of anti-inflammatory cytokines in disease outcome. However, results are conflicting. Hida et al. showed that there was increased expression of *IL-12* mRNA in the majority of patients with duodenal ulcer (Hida et al., 1999). In contrast, Itoh et al. showed that the Th response in GU patients was more

polarized towards Th1 than in DU patients (Itoh et al., 1999). In our study, no significant correlation was found between Th subset response and peptic ulcer disease. However, the levels of *IFNγ* mRNA were 10-fold higher in DU than non-peptic ulcer disease, implying that a true association could have been missed in our study due to the small number of patients we used (a type II error).

The trend in most patients in our study was towards a strongly polarised Th response to either Th1 or Th2 and this led us to examine factors which may drive Th differentiation. First we looked at the bacterial virulence factors CagA and VacA. Trends were toward higher levels of both *IFNγ* and *IL-12* mRNA in tissues infected with *cagA*+ than *cagA*- strains, suggesting a possible role for CagA in the polarization of Th responses. This seems reasonable because of the epidemiological relationship between CagA and gastric diseases (Stephens et al., 1998). Neither our study nor the UK study showed significant correlations between VacA and Th cell polarization.

The association between smoking and increased risk of gastric cancer is well established, but the mechanism underlying this is unclear. In an epidemiological study conducted in Portugal, smoking was shown to be associated with gastric intestinal metaplasia, a precancerous condition (Peleteiro et al., 2007). In other studies, epidemiological evidence linking smoking and gastric cancer has been described (De Stefani et al., 1998, Gonzalez et al., 2003). We hypothesised that smoking may be associated with a more pro-inflammatory Th1 response, potentially explaining these associations. In accordance with this hypothesis, we found that levels of *IFNγ* mRNA

were higher in smokers than non-smokers in Kurdistan region, Iraq. These results were confirmed by studying IgG subclasses in sera taken from Kurdish Iraqi subjects. To confirm our results in a different population, we studied the levels *IFN*γ mRNA in UK patients, and again found that the levels of *IFN*γ mRNA were higher in smokers than non-smokers. We speculate that smoking may direct the immune response toward Th1 biased immunity and increase IFNγ and other proinflammatory Th1 cytokines that may predispose to pre-malignant pathology including atrophy and metaplasia.

A strong association between sex hormones and Th differentiation has been found in several studies (Ansar et al., 1985). In a study conducted in female gerbils, estradiol treatment increased the intensity of inflammation in response to *H. pylori* infection, whereas progesterone decreased it (Saqui-Salces et al., 2006). In a study conducted in mice, estradiol exerted a protective effect against *H. pylori*-induced gastric cancer (Ohtani et al., 2007). In another study conducted in Japan, interleukin-10 production from human PBMCs was significantly higher in women than in men (Ono et al., 2005). In our study, the levels of *IL-4* and *IL-10* mRNA expression were higher in Iraqi females than males. However, IgG subclass ELISAs could not confirm these results. This might be because Th2 responses have only a small effect on IgG subclass levels (Fox JG et al., 2000, Mitchell et al., 2002). Also, the association of gender with cytokine mRNA expression was not confirmed in our UK population. However, this might have been an artefact due to the small sample size. More studies are needed to explore the correlation between female gender and predominant Th2 response.

This study has several limitations: in particular the sample size. However, this is likely to hide true positive associations rather than produce false positives. Additionally, when using RT-PCR to investigate immune responses, the levels of cytokine mRNA are measured, but this does not always accurately reflect levels of cytokine protein expression and does not define the cellular sources of the cytokines. However, the absolute levels of cytokines, especially IFNγ, are likely to be the crucial determinant for the disease process. For example, intestinal metaplasia developed in the stomach of mice transfused with IFNγ, even without *H. pylori* infection (Cui G, 2003). In addition, Robinson et al. have shown previously that cytokine RT-PCR and flow cytometry (which is an accurate method for determining numbers of Th1 and Th2 cells present) largely agree when performed on gastric biopsy specimens (Robinson et al., 2008). In our study, we did not depend completely upon RT-PCR: our smoking results were confirmed by studying the levels of IgG subclasses in serum.

To conclude, low Th1 responses may contribute the low cancer rate in Kurdistan region, Iraq. The association between high Th1 response and smoking suggests that smoking may promote a pro-inflammatory Th1 response: such a response is known to induce precancerous gastric atrophy and metaplasia. Our study is robust in supporting the association by two separate methodologies and in two distinct populations. The association between female gender and a higher IL-4 (Th2) response in Kurdistan region, Iraq may help to explain the lower gastric cancer incidence in women. However, these results need further confirmation.

5. *Helicobacter pylori dupA* is Naturally Polymorphic and Induces Cytokine Secretion from Peripheral Blood Mononuclear Cells but not from Gastric Epithelial Cells

Abstract

The duodenal ulcer promoting gene (*dupA*) is a recently discovered *Helicobacter pylori* virulence factor, shown to stimulate with interleukin-8 (IL-8) secretion by epithelial cells and to be associated with duodenal ulcer. Nucleotide sequencing of the *dupA* 3' region from 32 strains showed that *dupA* commonly had additional single base insertions or deletions that either truncated or extended the open reading frame. We have therefore classified *dupA* into two main groups: the common extended ORF within *jhp0917-19* (*dupA1),* and *dupA* with an early stop codon to truncate the ORF (*dupA2).* We aimed to study effects of DupA polymorphisms on inflammatory responses, examining cytokine responses of epithelial cells and blood mononuclear cells. Four pairs of parental (2 *dupA1* strains and 2 *dupA2* strains) and *dupA* mutant *H. pylori* strains, all possessing a functional *cag* pathogenicity island, were co-cultured with human gastric epithelial cell lines (AGS, ST16, MKN45, and MKN28) and peripheral blood mononuclear cells (PBMCs) from 5 *H. pylori*-negative healthy donors. IL-8, IL-12, IL-4, IL-10, interferon-γ (IFNγ), and tumour necrosis factor-α (TNFα) concentrations in co-culture supernatants were assayed by ELISA. *cagE* and *dupA* double mutants were constructed in two strains and tested in co-culture. RT-PCR was performed to measure the level of *IL-12* and *IL-10* mRNA in biopsies taken from patients infected with *dupA*-positive *H. pylori* (n=13) and *dupA*-negative *H. pylori* (n=22). Whereas strains possessing *cagE* induced significantly more IL-8 secretion from gastric epithelial cells than those lacking this locus, there was no significant difference between strains that possessed or lacked *dupA* irrespective of its polymorphisms. While disrupting *dupA1* significantly reduced IL-12, IFNγ, TNFα and IL-8 production by PBMCs, disrupting *dupA2* showed no effect on cytokine secretion. *cagE* mutation had no effect upon cytokine production by PBMCs. There were trends towards higher levels of *IL-12* and *IFNγ* mRNA in biopsies taken from patients infected with *dupA*-positive *H. pylori*, although these differences were not statistically significant. I therefore hypothesise that *dupA1* is a major determinant of *H. pylori*-induced mononuclear cell-associated inflammation.

5.1 Introduction

Helicobacter pylori infects approximately half the world's population and is the major cause of gastric and duodenal ulceration and gastric adenocarcinoma. *H. pylori* strains possessing the *cag* pathogenicity island (PaI) induce high levels of pro-inflammatory interleukin (IL)-8 secretion *in vitro* and also in the stomach *in vivo* (Atherton et al., 1995, Censini et al., 1996, Crabtree and Lindley, 1994, Keates et al., 1997). The *cag* PaI encodes a type IV secretion system that facilitates the translocation of CagA and soluble components of the *H. pylori* cell wall peptidoglycan into the host cell cytosol (Asahi et al., 2000, Odenbreit et al., 2000, Segal et al., 1999, Stein et al., 2000, Viala et al., 2004). Both translocated molecules activate signalling pathways that result in the activation of NF-κB and the secretion of IL-8 from the cell (Brandt et al., 2005, Viala et al., 2004). Although the *cag* PaI is the major pro-inflammatory *H. pylori* virulence factor, OipA has also been shown to contribute to IL-8 secretion (Kudo et al., 2005, Lu et al., 2005b, Yamaoka et al., 2002a, Yamaoka et al., 2000, Yamaoka et al., 2006), although some have failed to show this (Akanuma et al., 2002, Ando et al., 2002, Brandt et al., 2005, Dossumbekova et al., 2006, Odenbreit et al., 2002).

More recently, a novel virulence gene, *dupA*, has been identified that may also play a role in IL-8 secretion (Lu et al., 2005a). *dupA* has homology to *virB4* and comprises two overlapping genes (*jhp0917* and *jhp0918*) in genome sequence strain J99, but Lu *et al.* (Lu et al., 2005a) showed that *dupA* formed a single open reading frame (ORF) in *H. pylori* strain C142, and 23 other strains, due to a single nucleotide insertion (at position 1386), and they showed that the presence of *dupA* was significantly associated

with duodenal ulceration, but negatively associated with gastric cancer in populations from South Korea, Japan, and Colombia, and appeared to increase IL-8 secretion by epithelial cells. Populations from northern India and Iraq also exhibited a significant association between the presence of *dupA* and duodenal ulceration, although no patients with gastric cancer were included in these studies (Arachchi et al., 2007, Hussein et al., 2008a), whereas populations from Brazil and Iran had no significant association between *dupA* prevalence and ulceration or cancer (Gomes et al., 2008, Hussein et al., 2008a). We have recently found, however, that *dupA* was not significantly associated with duodenal ulceration in populations from Belgium, South Africa, China, and the USA, but was significantly associated with gastric cancer development (Argent et al., 2007). This association with duodenal ulceration in some populations and gastric cancer in others is similar to the association of the *cag* PaI with disease The *cag* PaI is thought to induce increased gastric inflammation largely through increasing IL-8 secretion from epithelial cells.

High levels of chemokines (IL-8, GRO alpha, ENA-78, RANTES, MCP-1) (Hida et al., 1999, Shimoyama et al., 1998) have been detected in the *H. pylori* infected gastric mucosa along with pro-inflammatory cytokines such as TNFα, IL-1β, IL-6, IL-7, IL-12 and IL-18 (Bauditz et al., 1999, Dzierzanowska-Fangrat et al., 2008, Yamaoka et al., 1995, Yamaoka et al., 1997). Increased levels of many of these factors are predicted to increase the risk of disease (El-Omar et al., 2003, Macarthur et al., 2004). Anti-inflammatory cytokines such as IL-10 and transforming growth factor-β have also been detected (Hida et al., 1999, Lindholm et al., 1998). *H. pylori* has been shown to

disrupt the tight junctions of gastric epithelial cells *in vitro*, possibly through intraepithelial injection of CagA protein (Amieva et al., 2003). Such effects *in vivo* would open the paracellular route for bacterial penetration deep into the mucosa. Recent investigations by Necchi *et al.*, suggested that *H. pylori* penetrates normal, metaplastic, and neoplastic gastric epithelium *in vivo*, intracellularly or interstitially to generate a strong immune-inflammatory response and promote gastric carcinogenesis (Necchi et al., 2007). Such reports indicate that studying the interaction of *H. pylori* with epithelial cells is only part of the story. The responses of immune and inflammatory cells should also be investigated when studying the role of *H. pylori* virulence factors. This prompted us to study the sequence of *dupA* in detail, determine the influence of *dupA* on the immune response, studying the responses of gastric epithelial cell lines and leukocytes *in vitro* as well as examining cytokine expression in gastric biopsies of donors infected with *dupA*+ and *dupA*- *H. pylori* strains.

5.2 Materials and Methods

All chemicals and reagents were obtained from Sigma-Aldrich (Poole, Dorset, UK) unless otherwise stated.

5.2.1 Patients

Biopsy specimens were donated by patients undergoing upper gastro-intestinal endoscopy at Azadi teaching hospital Dohuk, Kurdistan region, Iraq with approval from Hospitals Ethics Committee (35 *H. pylori*-infected patients: 13 infected with *dupA*+ strains and 22 infected with *dupA*- strains). The mean age ± standard deviation of the patients from Iraq was 37±18 years. During gastroscopy, 2 biopsy samples were taken from the antrum. One was placed in 1 ml of isosensitest broth (Oxoid, Basingstoke, UK) containing 15% (v/v) glycerol and stored in liquid nitrogen for future isolation of *H. pylori*. The other was placed in RNAlater solution, and preserved for mRNA analysis. DNA was extracted directly from the biopsy specimens using a DNeasy Blood and Tissue Kit (QIAGEN, Crawley, West Sussex, UK) and used for PCR-based *H. pylori* typing as previously described (Hussein et al., 2008a).

5.2.2 Culture of *H. pylori*

Each biopsy specimen was spread on both sheep blood and Dent agar plates, then incubated under microaerobic conditions generated by a Campypack (Becton, Dickinson and company) in an anaerobic jar at 37°C for 2 to 4 days. The organisms were identified as *H. pylori* by colony morphology; Gram stain; and urease activity.

Cultures were harvested and stored in 1 ml nutrient broth containing 15% glycerol at -80°C.

5.2.3 Culture of *Escherichia coli*

Single colonies of DH5α (White et al., 1988) were inoculated into Luria-Bertani (LB) (Oxoid) media supplemented with appropriate antibiotics where necessary. Broths were then incubated overnight at 37ºC with shaking at 200 rpm. The bacteria were then used for plasmid minipreps.

5.2.4 PCR Amplification and Nucleotide Sequencing of *dupA* (performed by Richard Argent)

Initially, the full-length *dupA* gene (based on the nucleotide sequence from *H. pylori* strain C142 - GenBank accession number AB196363) was PCR amplified from the 8 strains used to construct isogenic mutants (strains 93-67, 93-68, A101, AB21, AB31, AB43, J178 and J123), using primers *dupA*-WXF (5'-GATATACCATGGATGAGTTCYRTAYTAACAGAC) and DAR1. The amplified genes were sequenced on both strands using BigDye1.1 on a Perkin-Elmer ABI Prism 3130 fluorescent DNA analyser. It was apparent from the sequence analyses that some *dupA* genes had single nucleotide insertions close to the 3' end of the gene that extended the open reading frame. Therefore, a reverse primer was designed within *jhp0919* to include the next predicted stop codon, based on the nucleotide sequence from *H. pylori* strain J99. This reverse primer, JHP0919R2 (5'-GCCCACCAGTTGCAAAAACAAATGAAC) was successfully used with primer

*dupA*918F (Argent et al., 2007) to PCR amplify the extended region. Nucleotide sequencing of additional strains was performed following PCR amplification of the "extended" *dupA* gene using primers *dupA*-WXF and JHP0919R2.

5.2.5 Construction of Mutants

According to the sequence results, seven strains (J123 with a complete 1839 bp ORF similar to strain C142 as used by Lu *et al.* (Lu et al., 2005a); AB21, AB31, AB43 and 93-68 with a complete ORF extending into *jhp0919*; 93-67 and A101 with a stop codon in *jhp0917* truncating the ORF) were chosen for the construction of isogenic *dupA* insertion mutants (Figure 5.1). Five of these possessed a functional *cag* PaI (strains 93-67, J123, A101, AB21, and AB31) and two lacked this locus (AB43 and 93-68). I also constructed isogenic *cagE* insertion mutants in *dupA*-deficient mutants of strain 93-67 and AB31.

1 kb of *dupA* was amplified from *H. pylori* strain 93-67 using primers DAF1 (5'-GACGATCTATTGAATTTATACGCTG) and DAR1 (5'-TTAAATACTCTTCCTTATAAGTTTCTTGG) and PCR products were purified and cloned into pGemT-Easy (Promega, Southampton, UK) to form pGEM®-9367. pGEM®-9367 was transformed into the DH5α strain of *E. coli*. This was followed by extracting the plasmid and inverse PCR was used to engineer XhoI and XmaI sites into *dupA* using primers NHDA2R (5'-CTATATCCCGGGAGCTTGAAAATTGAAAAAGTG) and NHDA3F (5'-CAATACCTCGAGGCTAATACAGATGGTGGT). The chloramphenicol

resistance cassette from pBSC103 (Wang and Taylor, 1990) was excised using XhoI and XmaI and ligated into the *dupA* genes via the same sites to create plasmid pCDA723 (disrupted *dupA* from strain 93-67). Restriction products were run on agarose gels and the 4kb fragment containing *dupA* and the 1kb catR fragment were extracted and purified. *H. pylori* strains were transformed with plasmids by natural transformation and allelic exchange mutants were selected by marker rescue on blood agar plates (Oxoid) containing 30 μg/ml chloramphenicol. Disruption of *dupA* and confirmation of double crossover were determined by restriction digestion and determination of product size (Figure 5.2).

5.2.6 Gel Extraction and Purification of PCR Product

A QIAEX II Agarose Gel Extraction Kit (QIAGEN, Crawley, UK) was used for extraction of PCR products from 1.5% agarose gel, according to the manufacturer's instructions. A QIAquick PCR Purification Kit (QIAGEN, Crawley, UK) was used for the purification of the PCR product, according to the manufacturer's instructions. PCR fragments were purified from primers, nucleotides, polymerases, and salts by binding the DNA to a silica-gel membrane in the presence of high salt while contaminants pass through the column.

5.2.7 Ligation of DNA Fragments

PCR products were cloned into vector DNA (pGEM®-T Easy vector) at an approximate molar ratio of 3:1 insert to vector DNA. Reactions were carried out in a final volume of 20 μl in the presence of 3U T4 DNA ligase and buffer containing adenosine-5'triphosphate (ATP). Ligation reactions were incubated overnight at 4°C.

5.2.8 Isolation of Plasmid DNA

A QIAprep Spin Miniprep Kit (QIAGEN, Crawley, UK) was used to extract plasmid DNA following the manufacturer's instructions. Plasmid DNA was purified from 5 ml overnight cultures of *E. coli* in LB medium by binding the DNA to a membrane, washing to remove contaminants and eluting in water.

5.2.9 Growth Curves

H. pylori strains were cultured for 48 h on blood agar plates and the growth of 2-3 plates harvested then resuspended in 3 ml Brucella broth (Sigma). The cell pellets were equalised according to an optical density (OD) of 0.1 at 600nm. A 75 cm^2 tissue culture flask containing 50 ml sterile Brucella broth was then inoculated with 500 μl of the bacterial suspension and incubated at 37°C in a microaerobic environment within a MACS-VA500 workstation. At various time points a 1 ml sample was taken from the flask and the OD measured at 600 nm.

5.2.10 Eukaryotic Cell Culture

AGS and ST16 cell lines were grown in nutrient mixture F12 Ham (Invitrogen, Paisley, UK) containing 10 % (v/v) foetal bovine serum (FBS). MKN45 cells were grown in RPMI1640 medium, supplemented with 10% FBS, and 200mM l-glutamine (Invitrogen). MKN28 cells were grown in DMEM/Ham's F12 mixture, with 10% FBS and 200mM l-glutamine. All cell lines were cultured at 37°C in a 5 % CO_2 humidified atmosphere.

5.2.11 Peripheral Blood Mononuclear Cell (PBMC) Isolation

20 ml blood samples from 5 *H. pylori*-negative volunteers were collected into K_3EDTA vacutainer tubes (Greiner Bio-One GmbH, Kremsmunster, Austria). PBMCs were immediately purified by density gradient centrifugation using Histopaque 1077 and cultured in RPMI1640 medium supplemented with 10% FBS and 200mM l-glutamine.

5.2.12 Co-culture of Cells with *H. pylori*

As described previously**,** AGS, ST16, MKN45, MKN28 cells (Reyes-Leon et al., 2007) were seeded into multi-well tissue culture plates and grown to 80-90% confluence. PBMCs were plated at 2 x 10^6 cells per well in 24-well plates. *H. pylori* strains, grown on blood agar for 24 h, were harvested into 5 ml cell culture media and the OD standardized to 0.1 at 600nm. *H. pylori* was added to cultures at a bacteria:cell ratio of 100:1 for AGS, ST16, MKN45 and MKN28 cells and 20:1 for PBMCs. Co-culture assays were performed in 5 replicates per condition.

Supernatants were removed after 6 and 48 hours of culture for assay of cytokines by ELISA.

5.2.13 Cytokine ELISA

Cytokine responses in co-culture supernatants was measured using human IL-8 (R&D Systems DuoSet), IL-10, TNFα, IFNγ (eBioscience) and IL-12 (Autogen Bioclear) ELISA kits. All samples were assayed in 5 replicates at a 1/2 dilution with a standard curve on each plate. Assay sensitivity was determined as the mean plus three times the standard deviation of six control wells containing no cytokine. The optical densities were measured using a microplate reader at 450nm and 620nm. The cytokine concentration in each sample was calculated by reference to the standard curve.

5.2.14 cDNA Synthesis

Total RNA was extracted and purified from gastric biopsies using the QIAGEN kit (QIAGEN, Crawley, UK). Samples were quantified using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies Wilmington, DE, USA). The synthesis of cDNA from 50 ng RNA was conducted by using SuperScript II reverse transcriptase (Invitrogen) with oligo(dt) primer as per the manufacturer's instruction.

5.2.15 Real Time PCR

RT-PCR was performed on a Rotor-Gene 3000 (Corbett Research) by using the DyNAmo HS SYBR green qPCR kit (GRI). A volume of 20 µl was used for the amplification reaction. No-template controls were included in each cycle and a melting

curve analysis was performed to ensure the specificity of primers. To ensure that products were not amplified from contaminated genomic DNA, first stage RT-PCR samples produced in the absence of reverse transcriptase from each RNA template were tested in parallel. Samples were run in duplicate and results were analysed according to the method described by Pffafl (Pfaffl, 2001). Relative expression levels of the gene of interest *IL-4, IL-10, IFNγ,* (Lundgren A et al., 2003, Robinson et al., 2008) and *IL-12* were determined by normalising against a housekeeping gene (*GAPDH*) (Walker et al., 2003), and then comparing this value to the normalised level to cDNA made from a pooled RNA preparation from 5 *H. pylori*-negative patients to obtain a fold difference. A commercial pooled human cDNA standard (BD Biosciences) was included as a positive control in all assays. Amplification of *IL-4*, *IL-10* and *IFNγ* was carried out over 45 cycles of 15 seconds at 95^{o}C, 30 seconds at 61^{o}C and 30 seconds at 72^{o}C. IL-12 amplification was carried out over 45 cycles of 15 seconds at 95^{o}C, 30 seconds at 62^{o}C and 30 seconds at 72^{o}C. Commercial primers were used for human IFNγ (SuperArray RT, Tebu-Bio, Peterborough, UK). Other PCR primer sequences are shown in Table 4.1. Efficiencies of the IL-12 and IL-10 RT-PCR were tested using serial dilutions of a commercial human cDNA preparation (BD Biosciences).

5.2.16 Statistics

Data were analyzed using the Minitab 15 software program. A Student's t test was used to compare the growth rate between wild-type and parental strains of *H. pylori* and to compare the cytokines responses in co-culture supernatant. Statistical tests of

unpaired data were carried out using the Mann-Whitney U-test. Box and whisker plots are shown, where boxes represent the range of the first and third quartiles, the median values are shown as horizontal lines within the bars and the whiskers depict the lowest and highest observations within 1.5 times the first to third inter-quartiles ranges. A significant difference was taken at $p \leq 0.05$ unless otherwise stated.

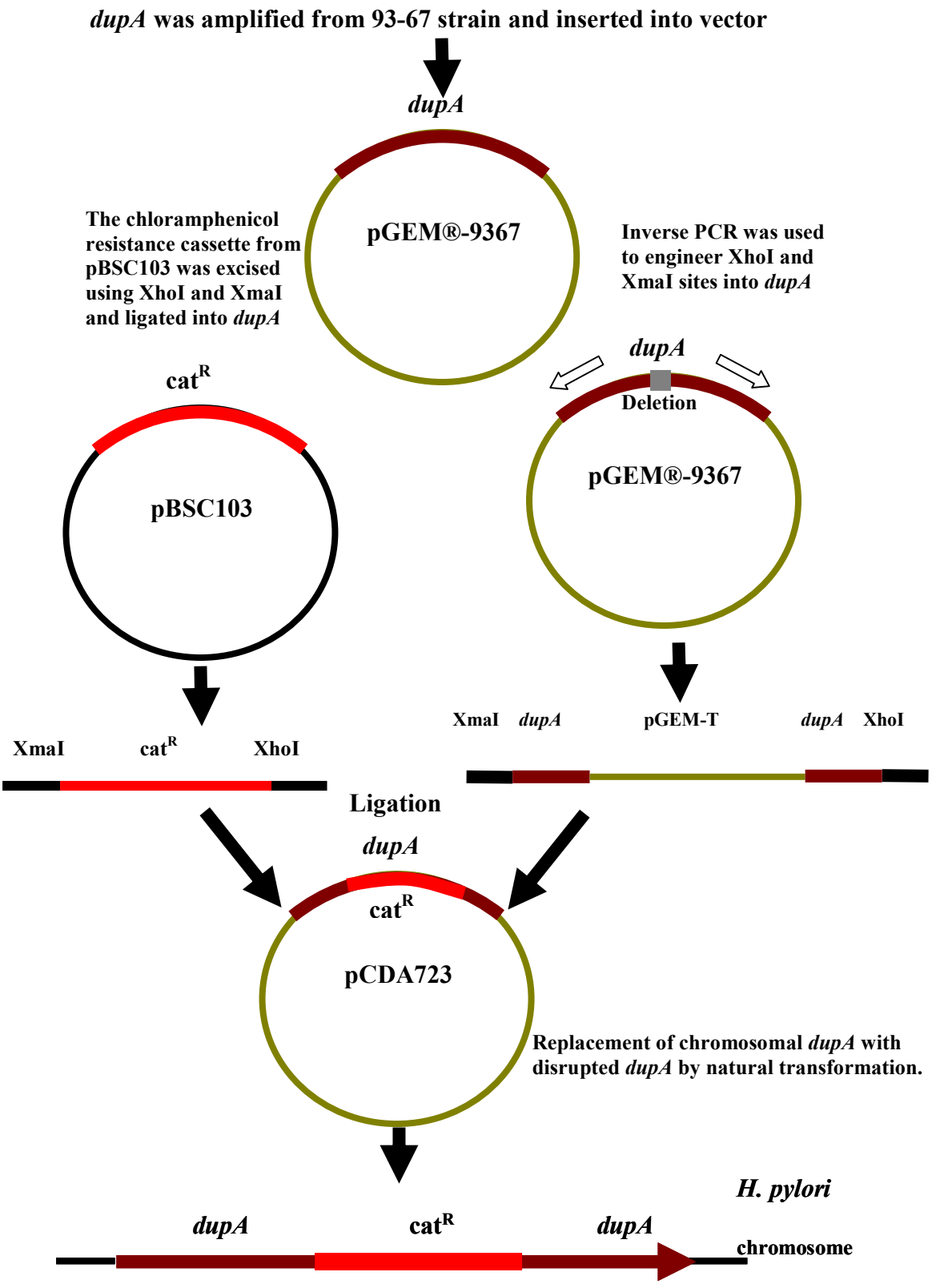

Figure 5. 1 Construction of *dupA* mutation.

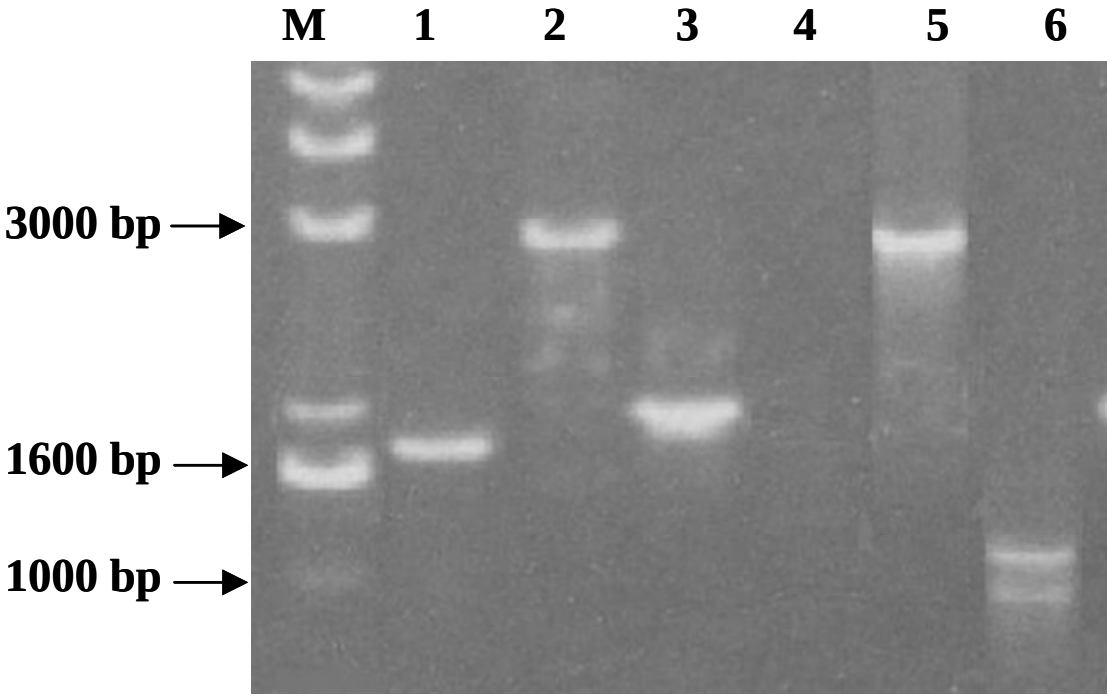

Figure 5. 2 Characterisation of *H. pylori* mutant using PCR and restriction enzymes

1. Wild-type AB31: *dupA* amplification using *dupA* flanking primers (amplifying most of the *dupA* ≈1800bp)
2. Mutant-type AB31: *dupA* amplification using *dupA* flanking (amplifying most of the *dupA* and chloramphenicol cassette ≈2800bp)
3. Mutant type AB31: chloramphenicol cassette amplification using one primer located within chloramphenicol cassette (Wang and Taylor, 1990) and the other located out side the Chloramphenicol cassette (amplifying chloramphenicol cassette and part of *dupA* ≈ 2000bp)
4. Wild-type AB31: chloramphenicol cassette amplification using chloramphenicol cassette flanking primers (No product)
5. Mutant-type AB31: *dupA* amplification using *dupA* flanking primers (amplifying most of the *dupA* and chloramphenicol cassette ≈2800bp)
6. Mutant-type AB31: *dupA* amplification product after 2 hours incubation with XhoI and XmaI restriction enzymes showing the cut chloramphenicol cassette (≈ 1000bp)

5.3 Results

5.3.1 Natural Polymorphisms within *dupA*

Lu *et al.* (Lu et al., 2005a) showed that *dupA* comprised two overlapping genes (*jhp0917* and *jhp0918*) in genome sequence strain J99, due to a single nucleotide deletion. This base (at position 1386) was present in strain C142, and in 23 other *dupA* genes sequenced by them, producing a continuous ORF of 1839 bp. We sequenced the entire *dupA* gene from eight further *H. pylori* clinical isolates (A101, J178, 93-67, J123, AB21, AB31, 93-68 and AB43) to determine if they possessed a 1839 bp gene *dupA*, and not two overlapping genes. We found that all strains indeed possessed a cytosine or thymidine at position 1386.

Surprisingly we found that the most common form of *dupA* (4/8 strains) incorporated nucleotide insertions (relative to strain C142) that extended the coding region beyond 1839 bp (Figure 5.3). All 8 strains had a variation at the 3' end of *dupA* that was not present in *dupA* from strains C142. The regions from nucleotide position 1738 to position 1800 were altered due to an adenine insertion (within a polyadenine stretch), and changed the deduced amino acid sequence from ICKNYFIFLIAMLVIEKSSMI (present in *dupA* from strains C142) to DLQELLYILDSNAGNRKILND in all 8 strains we sequenced. To determine the position of the stop codon in our extended *dupA* genes, the 3' region of *dupA* and the 5' region of the adjacent gene *jhp0919* was PCR amplified using primers *DupA*918F and JHP0919R2, and sequenced. This showed that the extended *dupA* was predicted to terminate after 1884 bp, 5 bp after the

start codon of *jhp0919*. Of the 8 *dupA* genes sequenced, three had single nucleotide deletions that introduced early stop codons.

Nucleotide sequencing of the *dupA* 3' region of 26 other strains from Belgium, South Africa, China, and USA (Argent et al., 2007), showed that 21 strains possessed the extended form of the *dupA* gene. Three strains (J133, GC5, and GC77) probably comprised two separate *jhp0917* and *jhp0918* genes, and two strains (32385 and J188) had truncated *dupA* genes of 1500 bp and 1635 bp, respectively. Taking into account all sequenced strains, the most common form of *dupA* (25/34 sequences in total) is the extended form. I have therefore classified *dupA* into two main groups: the common extended ORF within *jhp0917-19* (*dupA1),* and *dupA* with an early stop codon to truncate the ORF (*dupA2).* I consider that strains C142 (as used by Lu *et al.*(Lu et al., 2005a)) and J123, with a shorter *dupA* ORF in *jhp0917-18* could be a less common subgroup of *dupA1*.

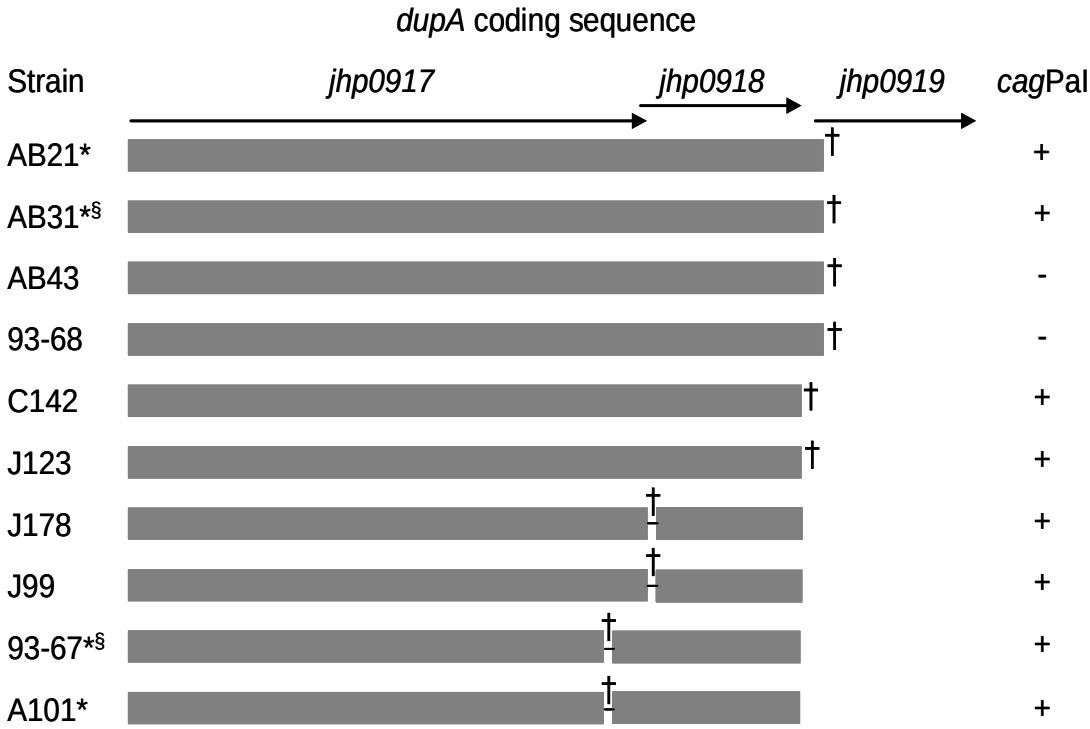

Figure 5. 3 Schematic representation of *dupA* gene.

Gene sequencing revealed a frame shift resulting in truncated *dupA* in the J178, J99, 93-67 and A101 strains, C142 and J123 carry the complete open reading frame and AB31 and AB12 carry the extended complete open reading frame. * indicates strain from which *dupA* mutant was derived, § indicates *cagE* also mutated,† indicate the presence of stop codon

5.3.2 Growth Comparisons of Wild-type and *dupA* Mutant *H. pylori* Strains

To exclude any difference in growth rate between wild-type and mutant strains, the growth rate was compared. Student's *t* test showed no significant difference in the growth rate between any wild-type and mutant strain (Figures 5.4 shows an example for strain AB31).

5.3.3 Interleukin-8 Secretion from Gastric Epithelial Cells

5.3.3.1 Impact of *dupA* on Gastric Epithelial Cell IL-8 Responses (Richard Argent contributed partly to this part)

To assess quantitative effects of *dupA* and its polymorphisms on epithelial cell IL-8 production, parental and *dupA* mutants of strains 93-68, AB43, 93-67, AB21, AB31 and J123 were co-cultured with AGS cells before IL-8 quantification (Figure 5.5 A). No significant differences were observed between the paired wild-types and isogenic mutants. In order to test this more thoroughly, strains AB21, AB31 and J123 were co-cultured with the gastric epithelial cell lines ST16 and also MKN45 (as used by Lu *et al.* (Lu et al., 2005a)) (Figures 5.5 B and 5.5 C). A slight significant reduction in IL-8 was induced by the *dupA* mutant of J123 but this was only after 6 hours culture with ST16 cells (1.2-fold reduction; p=0.004). No difference was found after 48 hours with these cells, or at either time-point with MKN45 cells. No significant differences were observed with AB21 or AB31 strains.

These results conflicted with those published by Lu *et al.* (Lu et al., 2005a), who found a significant reduction in IL-8 release by MKN45 cells incubated with a *dupA*-deficient mutant of strain C142. In order to more thoroughly test this in our hands, a second set of *dupA* mutants derived from strains 93-67, A101, AB21 and AB31 were compared with the isogenic parental strains in co-cultures with MKN45 and AGS cell lines as before, but also MKN28 gastric epithelial cells (Figures 5.5 D-F). As before, for all pairs of *H. pylori* strains (wild-type and *dupA* mutant), the presence of *dupA* did not have any effect on IL-8 production by these gastric epithelial cell lines.

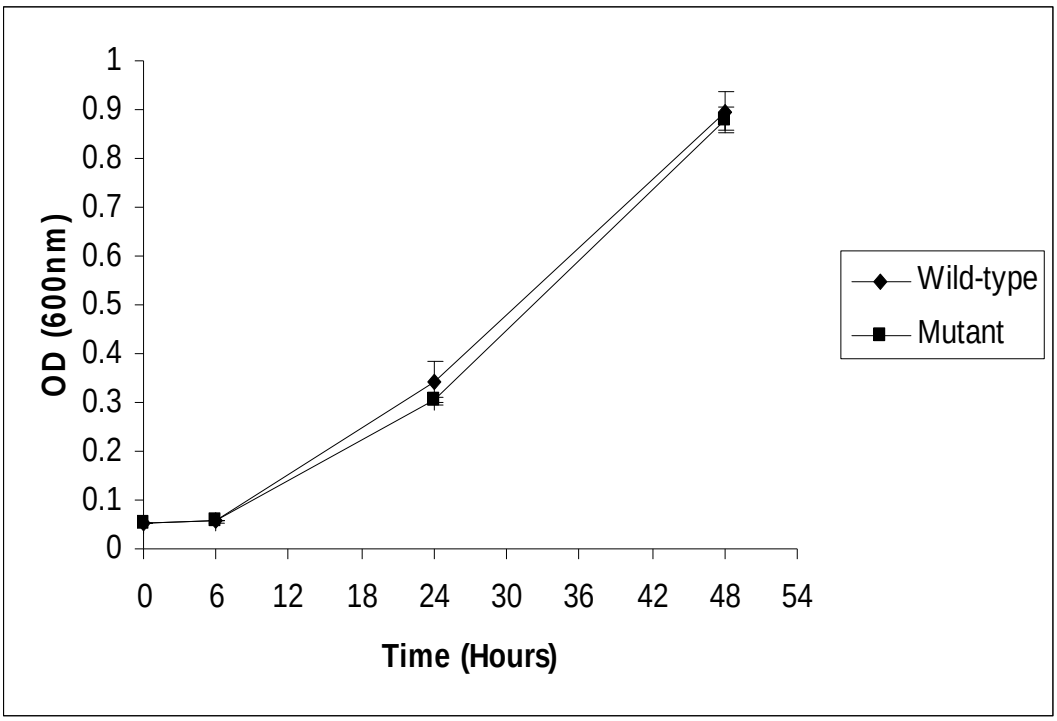

Figure 5. 4 Comparison of the growth rate of wild-type AB31 and its *dupA* isogenic mutant strain. Each point represents the mean of 3 reading. Error bars indicate standard deviation. OD: Optical Density.

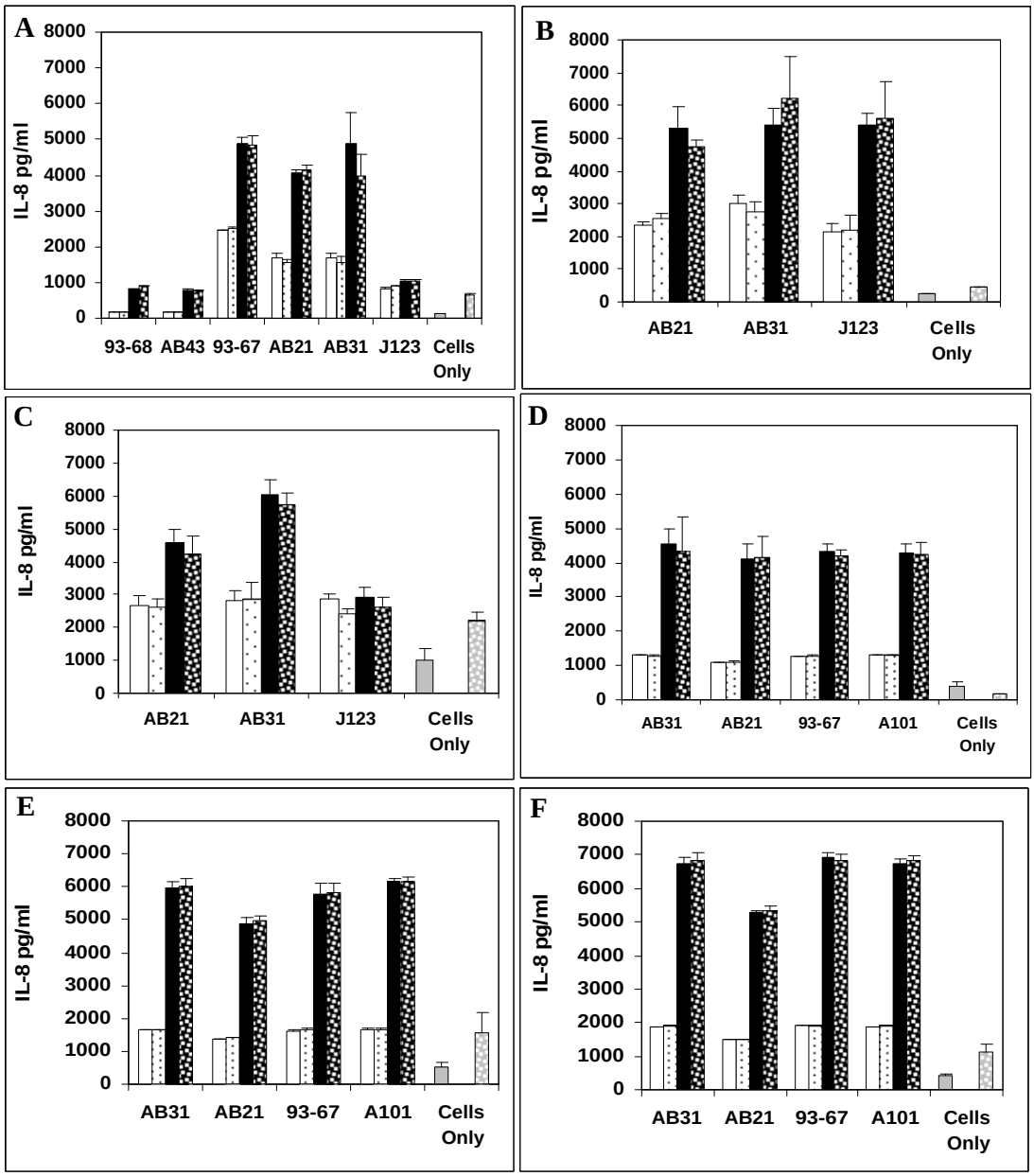

Figure 5. 5 Mean levels of IL-8 secretion from AGS (A & D), ST16 (B), MKN45 (C & E), and MKN28 (F) cells co-cultured with *H. pylori* strains (93-68, 93-67 AB43, AB21, AB31, J123, and A101) and their isogenic *dupA* mutants for 6 and 48 hours.

Data from 6 hours cultures is depicted as wild-type: plain white bar; isogenic *dupA* mutant: dotted white bar, and 48 hours as wild-type: solid black bar; isogenic *dupA* mutant: dotted black bar. The concentrations of IL-8 in the medium were determined by ELISA. Grey bars represent results obtained from cells cultured without *H. pylori* (6 hours: plain bar; 48 hours: dotted bar). Error bars indicate standard deviations.

5.3.3.2 Impact of the *cag* PaI on Gastric Epithelial Cell IL-8 Responses

As a control, and to prove that I could detect differences in IL-8 responses if they were present, I compared the effect of *dupA* mutation on IL-8 release by epithelial cells with disruption of the *cag* PaI. To do this we constructed *cagE* mutants in AB31 and 93-67 wild-type and *dupA* mutant strains (double and single gene knock-outs) and incubated them with AGS cells for 48 hours. The *dupA* mutants elicited the same level of IL-8 as the wild-type strains but, as expected, *cagE* mutation dramatically reduced the production of this chemokine (AB31: Wild-type: 4475±800 pg/ml; *dupA* mutant: 4079.1±159.8 pg/ml; *cagE* mutant 1041.7±106 pg/ml; *dupA* and *cagE* double knock-out 1162±130.9 pg/ml, 93-67: wild-type: 3945.8±136 pg/ml; *dupA* mutant: 3995.8±271 pg/ml; *cagE* mutant: 988±111 pg/ml; *dupA* and *cagE* knock-out 1050±62 pg/ml) ($p<0.01$ for all) (Figure 5.6).

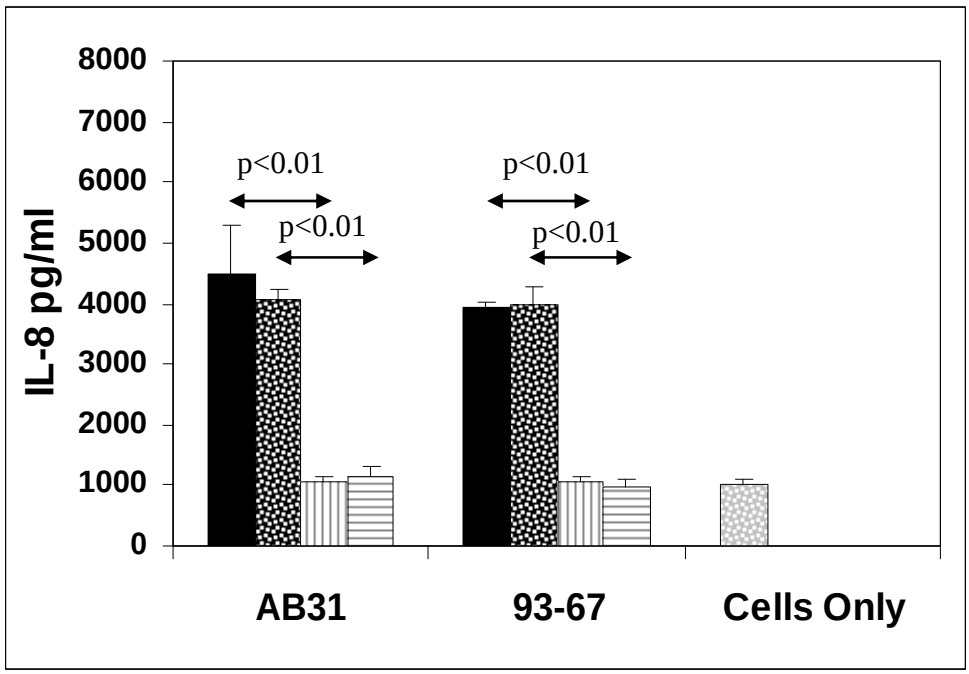

Figure 5. 6 IL-8 secretion from AGS cells at 48 hours.

Cells were co-cultured with *H. pylori* strains: wild-type (plain black bars), isogenic *dupA* mutants (dotted black bars), isogenic *cagE* mutants (vertically striped bar) and isogenic double (*cagE* and *dupA*) mutants (horizontally striped bar) for 48 hours before the concentration of IL-8 in the medium was determined by ELISA. Error bars indicate standard deviation.

5.3.4 Effect of *dupA* on Inflammatory Cytokine Responses of Mononuclear Cells

5.3.4.1 Effect of *dupA* on *H. pylori*-induced IL-12 Release

It has been reported that *dupA* is associated with increased risk of *H. pylori*-induced disease and that *dupA*+ strains induce increased inflammatory cell infiltration into the gastric mucosa (Arachchi et al., 2007, Hussein et al., 2008c, Lu et al., 2005a). I therefore hypothesised that *dupA* might interact with other categories of cells to stimulate higher levels of inflammation. There have been several recent reports concerning the penetration of *H. pylori* through the epithelium into underlying tissues (Aspholm et al., 2006, Necchi et al., 2007, Robinson et al., 2008) and effects of the bacteria and their virulence factors on immune and inflammatory cells are well-known (Amieva et al., 2003, Sundrud et al., 2004). I therefore investigated the effect of *dupA* on innate immune responses of human mononuclear cells, comparing inflammatory cytokine responses of PBMCs from five uninfected normal donors to *dupA* mutant and wild-type strains.

Co-culturing PBMCs with *H. pylori* strains and their isogenic *dupA* mutants for six hours resulted in minimal IL-12 production and consequently no significant differences were found between any of the strains and mutants. The IL-12 response of PBMCs cultured for 48 hours with wild-type strains carrying the extended ORF (*dupA1*) was significantly higher (Strain: mean±sd, AB31: 405.8±15.7 pg/ml, AB21: 447±42 pg/ml) than that induced by wild-type *H. pylori* carrying the truncated form (*dupA2*), (93-67 244±21 pg/ml, A101 271±60 pg/ml; $p<0.01$) (Figure 5.7). In addition, the mutants

derived from AB21 and AB31 induced only approximately half the IL-12 concentrations stimulated by the corresponding wild-types after 48 hours (AB31: wild-type 405.8±15 pg/ml; mutant 197±63 pg/ml; p=0.002, AB21: wild-type 447±42 pg/ml; mutant 162±7 pg/ml; p=0.001). No significant difference in IL-12 secretion was observed between *dupA2* wild-type and isogenic mutant strains, however (93-67: wild-type 244±21 pg/ml; mutant 235±41 pg/ml, A101: wild-type 271±60 pg/ml; mutant 271±65 pg/ml) (Figure 5.7).

5.3.4.2 Comparative Effects of *dupA* and the *cag* PaI on PBMC IL-12 Responses

To examine the strength of the effects on inflammation of *dupA* in comparison to the *cag* PaI, I incubated PBMCs with *cagE* and *dupA* single and double mutants that had been prepared from AB31 and 93-67 wild-type strains. Although it was clear that *dupA* mutation dramatically reduced the IL-12 response, unlike the results from co-cultures with gastric epithelial cells, disruption of *cagE* had no discernible effect on the level of IL-12 production (AB31: Wild-type: 481.7±58 pg/ml; *dupA* mutant: 293±99 pg/ml; *cagE* mutant 471.4±38 pg/ml; *dupA* and *cagE* double knock-out 281.8±37 pg/ml, 93-67: wild-type: 286±33.7 pg/ml; *dupA* mutant: 289.7±21.7 pg/ml; *cagE* mutant: 288.5±21.7 pg/ml; *dupA* and *cagE* knock-out 270±12 pg/ml) ($p<0.05$ for all) (Figure 5.8).

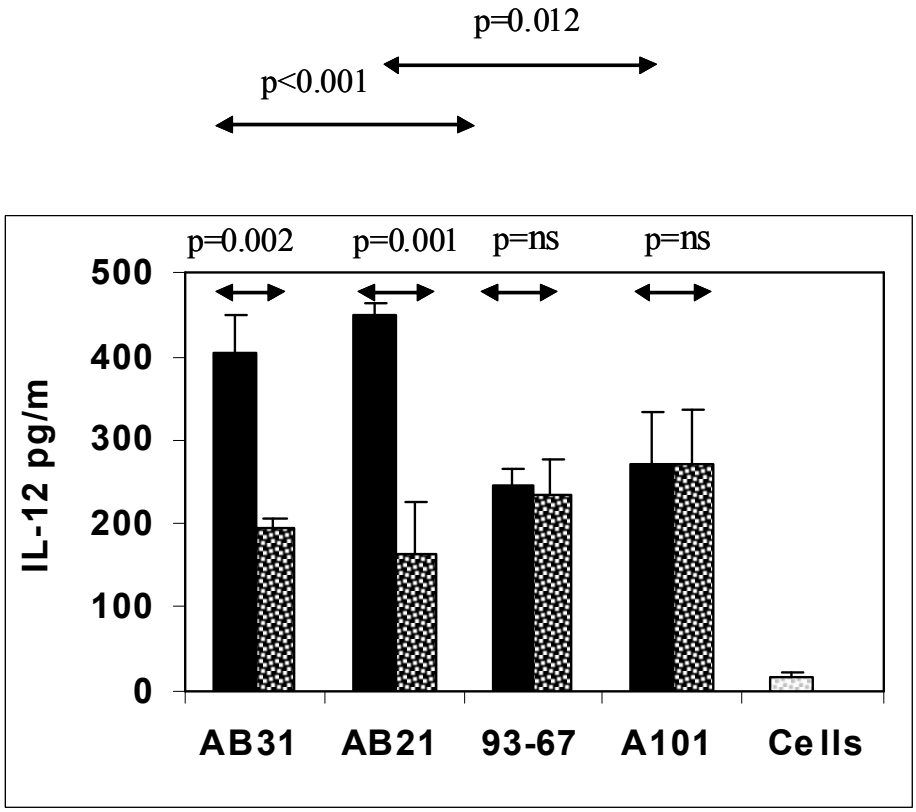

Figure 5. 7 IL-12 sectretion from PBMCs at 48 hours.

PBMCs were co-cultured with wild-type *H. pylori* strains (plain black bars) and their isogenic *dupA* mutants (dotted black bars) for 48 h. The dotted grey bar represents results obtained from cells only. Error bars indicate standard deviation. ns: no significant difference.

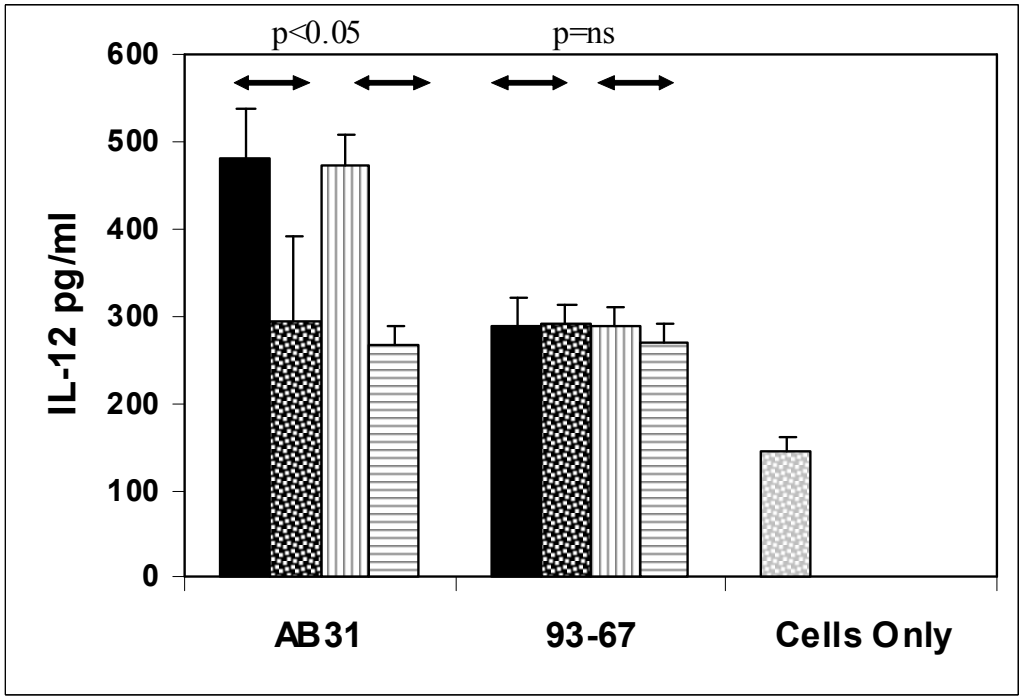

Figure 5. 8 IL-12 production from PBMCs at 48 hours.

PBMCs were co-cultured with *H. pylori* strains (e.g. AB31 in this figure): wild-type (plain black bars), isogenic *dupA* mutants (dotted black bars), isogenic *cagE* mutants (vertically striped bar) and isogenic double (*cagE* and *dupA*) mutants (horizontally striped bar) for 48 hours before the concentration of IL-8 in the medium was determined by ELISA. Error bars indicate standard deviation. ns: no significant difference.

5.3.4.3 Effect of *dupA* on the Production of Other Pro-inflammatory Cytokines by PBMCs

To confirm the IL-12 results, other cytokines (IFNγ, TNFα and IL-8) associated with Th-1 responses and inflammation were examined in PBMC cultures with strains AB31 and AB21 (*dupA1)*. The level of IFNγ was significantly higher in response to wild-type compared to *dupA* mutant strains (AB31: wild-type 96.9±8.6 pg/ml; mutant 71.9±6.9 pg/ml; p=0.007, AB21: wild-type 95.5±3.7 pg/ml; mutant 87.5±2.7 pg/ml; p=0.003) (Figure 5.9 A). Also in agreement with the IL-12 results, the level of TNFα was significantly higher in response to wild-type compared to *dupA* mutant strains (AB31: wild-type 1384.3± 24.5 pg/ml; mutant 1329.1± 30.3 pg/ml; p=0.02, AB21: wild-type 1802.3±30.7 pg/ml; mutant 1677±74.4 pg/ml; p=0.009) (Figure 5.9 B). Similarly, a significant reduction in the IL-8 response to AB31 and AB21 strain mutants was also found (AB31: wild-type 3043.2± 102 pg/ml; mutant 2815.6±46 pg/ml; p=0.02, AB21: wild-type 3756.4± 109 pg/ml; mutant 3518±43 pg/ml; p=0.02) (Figure 5.9 C). The differences in IL-8, TNFα and IFNγ responses attributable to *dupA* were less dramatic than observed for IL-12, but the trends were the same. No significant differences were found when the wild-types and mutants derived from 93-67 and A101 strains were compared for IFNγ, TNFα and IL-8.

Further assays were performed to investigate IL-10 and IL-4 responses as these reportedly correlate with reduced inflammation and disease *in vivo* (Fox JG et al., 2000, Robinson et al., 2008). No significant differences were observed with any of the strains, however (Figure 5.9 D&E).

5.3.5 Associations of *dupA* Status with Inflammatory Cytokine Expression *in vivo.*

To investigate whether the enhanced inflammatory responses induced by *dupA1* strains *in vitro* could be supported by observational *in vivo* data, real-time RT-PCR analyses were carried out on human gastric biopsy tissues (13 *dupA*+, 22 *dupA*-). In confirmation of the *in vitro* data, trends were found showing that in *dupA*+ *H. pylori*-infected gastric tissue, the levels of *IL-12* and *IFNγ* mRNA were higher than in tissues infected with *dupA*- *H. pylori* (Figure 5.10). These differences (1.2- and 1.6-fold respectively) did not achieve statistical significance, but I do not have information on the *dupA* sequence types from these samples. In *dupA*- *H. pylori*-infected compared to *dupA*+ *H. pylori*-infected tissues, there were 11 and 10-fold increased levels of *IL-10* (p=ns) and *IL-4* mRNA (p=0.06), respectively (Figure 5.10).

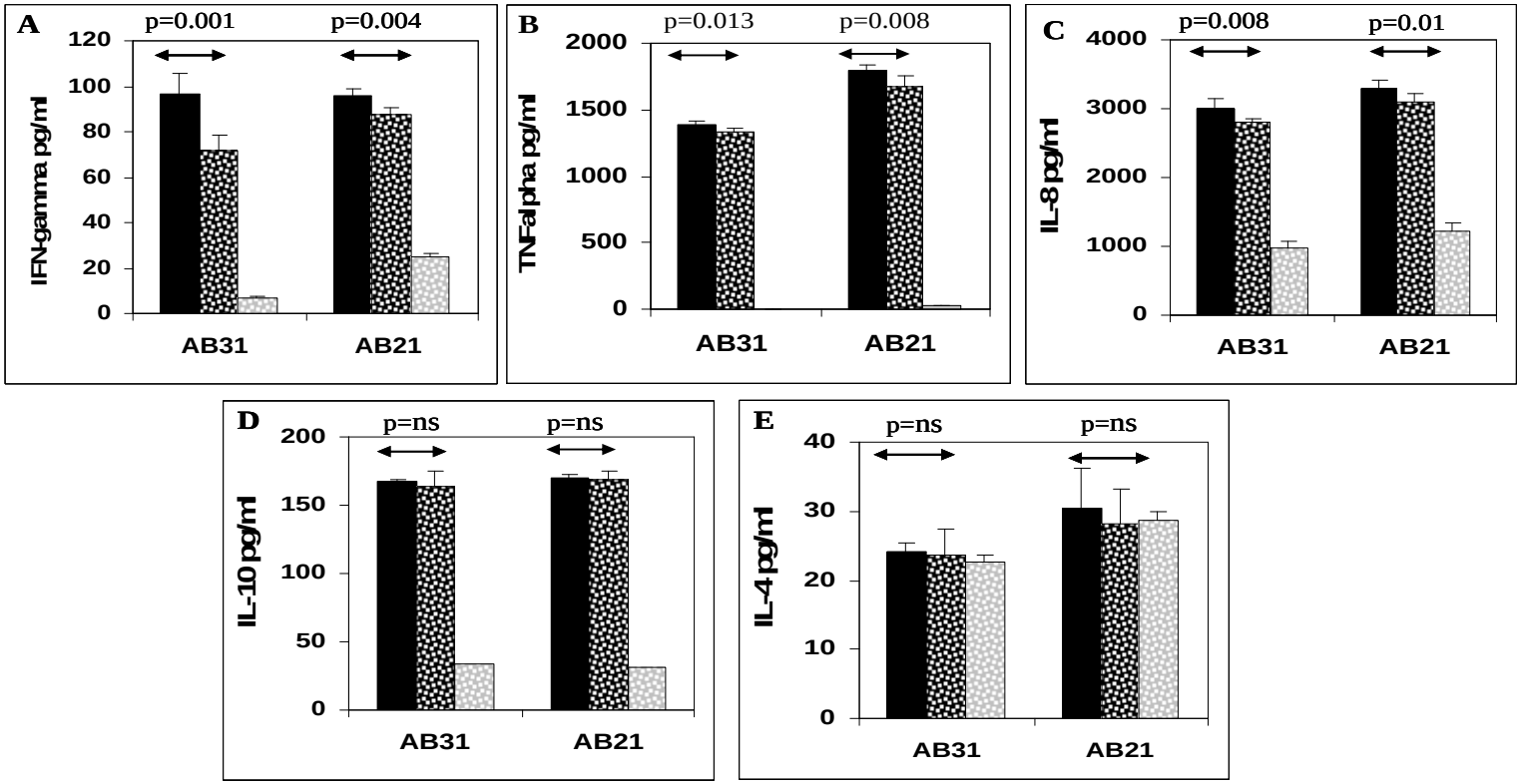

Figure 5. 9 Cytokines production from PBMC cells at 48 hours.

PBMC cells were co-cultured with *H. pylori* strains (AB31 and AB21). IFNγ (A), TNFα (B), IL-8 (C), IL-10 (D) and IL-4 (E) were measured by ELISA after co-culturing PBMCs with AB31 and AB21 *H. pylori* strains possessing *dupA* (plain black bars) and their isogenic *dupA* mutants (dotted black bars) for 48 hours. Dotted grey bars represent results obtained from cells only. Error bars indicate standard deviation. ns: no significant difference.

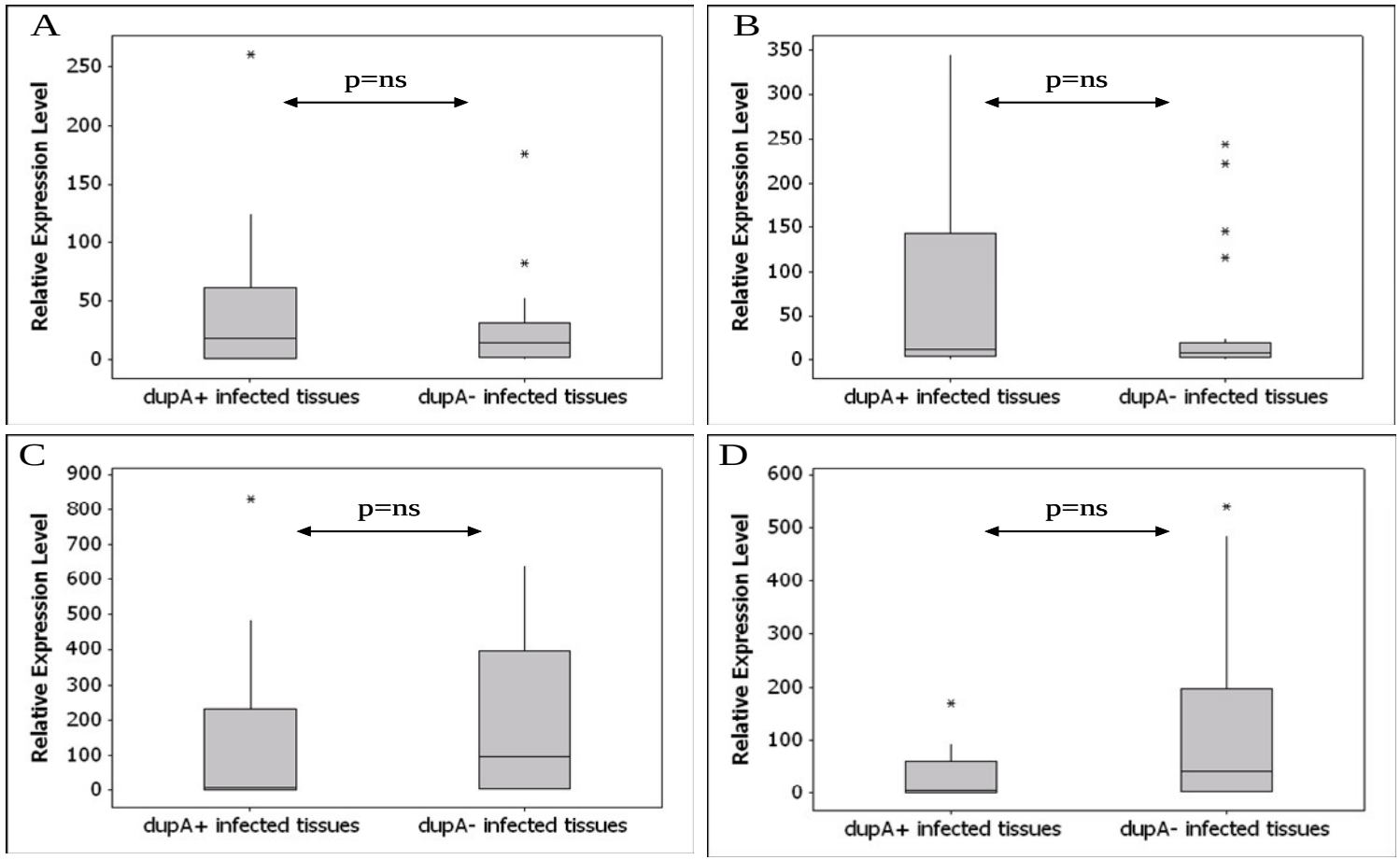

Figure 5. 10 Relative *IL-12* (A), *IFNγ* (B), *IL-10* (C) and *IL-4* (D) mRNA levels in gastric biopsies from 13 *dupA+ H. pylori* -infected tissues and 22 *dupA- H. pylori*-infected tissues. No significant differences were found * Outlier

5.4 Discussion

dupA is a recently described virulence factor associated with an increased risk for DU and reduced risk for gastric atrophy and cancer (Lu et al., 2005a). Studies from different parts of the world have confirmed or disputed these disease associations (Arachchi et al., 2007, Argent et al., 2007, Gomes et al., 2007, Hussein et al., 2008a, Lu et al., 2005a). Nucleotide sequencing of *dupA* revealed that this gene is naturally polymorphic and *dupA* polymorphisms might explain the regional dispute in results. Currently it is unknown which forms of the gene are expressed, which probably confounds such disease association studies. From our sequencing work and examination of published *dupA* sequence data I found that the most common form of the gene (*dupA1*) was extended to encompass *jhp0917, jhp0918,* and small part of *jhp0919*.

Co-culture of *H. pylori* strains with epithelial cell lines showed that there was no difference in the production of IL-8 induced by wild-type and the isogenic *dupA* mutant strains. This was tested rigorously using four different cell lines and seven panels of isogenic mutants, and by two independent members of our team. The mutant derived from J123 strain (with the same form of *dupA* as C142, used by Lu *et al.* (Lu et al., 2005a) previously) induced slightly less IL-8 than the parental strain but this was only observed with ST16 cells at the 6-hour time-point. The results with all other strains and cell lines did not detect a difference in IL-8 response due to the presence or absence of *dupA,* which is in conflict with the data of Lu *et al.* (Lu et al., 2005a). To validate our assays I performed comparisons of parental strains with *cagE*-deficient mutants and clearly showed that the *cag* PaI

stimulates IL-8 release from epithelial cells (as shown by many others (Brandt et al., 2005, Fischer et al., 2001, Viala et al., 2004)).

Many of the strains used in this study had a different form of *dupA* from that in C142 (used by Lu *et al.* (Lu et al., 2005a) to demonstrate associations with IL-8 secretion), which may explain the difference in our results, but our sequence data analyses showed that this is not a common *dupA* type. AB31 and AB21 strains (*dupA1)* had a variation at the 3' end of *dupA* that was not present in *dupA* from strains C142 or J99. The regions from nucleotide position 1738 to position 1800 were altered due to an adenine insertion (within a polyadenine stretch), and changed the deduced amino acid sequence from ICKNYFIFLIAMLVIEKSSMI (present in *dupA* from strains C142 and J99) to DLQELLYILDSNAGNRKILND in our strains. In support of our findings, Schmidt *et al.* (Schmidt et al., 2008) recently reported that, in a study of responses of AGS cells to clinical isolates from Malaysia and Singapore, the presence of *dupA* was not associated with elevated IL-8 release.

dupA+ strains have been reported to be associated with increased risk of gastro-duodenal disease. Ulceration and carcinogenesis are processes that are driven by inflammation (reviewed in (Atherton, 2006)) and increased levels of inflammatory cell infiltration have been reported with *dupA*+ strain infections (Lu et al., 2005a). I therefore examined the effects of *dupA* on leukocytes, which are another major source of inflammatory cytokines and chemokines in the infected gastric mucosa. Interestingly it was found that wild-type strains carrying *dupA*1 had a greater effect on the production of IL-12 by PBMCs than wild-type strains carrying the truncated

form of *dupA* (*dupA*2). Mutation of *dupA1* in strains AB31 and AB21 resulted in a significant reduction in IL-12 production compared to the parental strains. For other strains carrying *dupA*2, I could not find any differences in IL-12 induction which implies that the *dupA1* form of the gene is responsible for increasing levels of inflammation and disease. To confirm that presence of a *dupA1* gene is associated with increased stimulation of proinflammatory cytokines, the levels of IFNγ, IL-8 and TNFα were measured. It was found that the levels of these cytokines were also higher in response to wild-type compared with *dupA* isogenic mutant strains.

Correlations between virulence factors and immune responses have previously been investigated. Although the role of the *cag* PaI in inducing IL-8 from epithelial cells is clear, its importance in inducing inflammatory responses from inflammatory cells remains inconclusive. In a study conducted in China, it was shown that CagA+ strains were associated with the development of a T-helper 1 (Th1) human peripheral blood T-cell response (Wang et al., 2007). Such a response has been shown to be linked with premalignant gastric mucosal pathology and disease (Atherton, 2006, Cui G, 2003). Guiney *et al.* (Guiney et al., 2003) showed that mutagenesis of *cagE* reduced the IL-12 response of dendritic cells *in vitro*. In another study however, no relationship could be established between *cagA* expression and IL-12 production by monocytic cell lines (Odenbreit et al., 2001). Using PBMCs (consisting mainly of lymphocytes and monocytes) in this report I showed that *dupA1* plays an important role in stimulating IL-12 production whilst the *cag* PaI had no significant effect. *H. pylori* is able to simulate the immune response, and experiments in mice indicate that the quality of the T-helper response is critically important for eradication/persistence of the infection and

induction/prevention of *H. pylori*-mediated pathology (Akhiani et al., 2002, Lucas et al., 2001). This report puts forward the hypothesis that *dupA* plays an important role in increasing gastric inflammation and pathology by acting upon infiltrating leukocytes rather than epithelial cells, and increases the risk of disease by stimulating the development of a more pronounced Th1 immune response.

In support of this hypothesis, mucosal *IL-12* and *IFNγ* mRNA levels were higher in gastric biopsy specimens obtained from patients infected with *jhp0917-0918 dupA+* strains compared with those with *dupA-* strains. This trend did not achieve statistical significance however, probably because sequence data were not available to determine the polymorphic forms of *dupA*. The levels of *IL-10* and *IL-4* mRNA were lower in biopsy specimens taken from patients infected with *dupA+* than *dupA-* strains, indicating the presence of fewer Th2 and regulatory T-cells which suppress Th1-mediated immune and inflammatory responses. In this study, a clear trend towards a higher inflammatory Th1 and a lower Th2 response in *dupA+ H. pylori* infected tissues could be seen. Together, these findings suggest that a functional *dupA* gene plays an important role in enhancing inflammation possibly by skewing the immune response towards a Th1 response.

There are still many unknowns to be addressed concerning *dupA*. Almost all studies have so far merely compared disease incidence with *dupA* presence or absence by PCR genotyping, but our data have shown the gene to be highly polymorphic. To date it remains unknown which forms of the gene are expressed and active, although in this study I could only find a pro-inflammatory phenotype with *dupA1+* strains. The next step for our research will therefore be to generate an antibody

against recombinant DupA, as to our knowledge no one else has successfully prepared a specific antiserum (Yamaoka, personal communication). With DupA-specific serum I will then be able to assess which forms of the *dupA* gene are expressed as protein, and begin to address our hypothesis that *dupA1* is expressed and active whereas *dupA2* is not. I can also investigate the shorter *dupA1* forms, as found in C142 and J123. Such advances will allow us to further investigate associations between *dupA* status and gastric mucosal inflammatory cytokine responses *in vivo.*

We have not yet determined the mechanism by which DupA stimulates immune and inflammatory cells to secrete higher levels of cytokines. Bioinformatic analyses show that it has a high degree of homology with *virB4*-homologue ATPases such as *cagE* and *comB,* suggesting that it may be a component of an additional type IV secretion system. As such it is a possibility that *dupA* is a marker for the delivery of some other inflammatory factor to cells, which would make sense as *dupA* is not predicted to be secreted or surface exposed. As a first step in this direction, lysates from wild-type and *dupA*-deficient mutants must now be tested on PBMCs to ascertain whether live bacteria and therefore an active ATPase function is necessary to mediate pro-inflammatory effects. Further investigations are also necessary to determine which cell types within PBMCs are induced by DupA to secrete more IL-12. Since this factor is not secreted by lymphocytes we propose that monocytes are likely to be the source. Future experiments will use flow cytometry to determine this, and the effects of *dupA*+ strains and mutants on purified cell populations will then be determined.

In conclusion, we propose that the *dupA* gene is polymorphic and expression of the *dupA1* form may skew the differentiation of T-helper cells towards a Th1 response. This activity of *dupA* on inflammatory cells probably plays an important role in guiding the immune response to *H. pylori* infection, the level of inflammation and thus disease development.

6. Final Conclusion and Recommendations

6. Conclusions and Recommendations

Interestingly, in Kurdistan region, Iraq, despite a high prevalence of *H. pylori* infection, the prevalence of more serious *H. pylori* related disease and in particular gastric cancer is reported to be extremely low (GLOBOCAN, 2002). Indeed, examination of the medical records for the period 2005-2006 at the Sulaimanyia hospital (a hospital servicing 1million people in Sulaimaniya city) and Azadi hospital (a hospital servicing 800,000 people in Dohuk city) showed that only approximately 40 gastric cancers were diagnosed during that period in both cities. No cancer patient from Iraq was involved in this study because it was extremely difficult to find patients with gastric cancer. This did not affect my study because I am interested in *H. pylori* infection related factors which might contribute to the low cancer rate in this community.

First, I investigated the age-specific prevalence of *H. pylori* infection in Kurdistan region, Iraq. I showed that the seroprevalence of *H. pylori* in children of various age group was <6months:0%; 6-24months:27%; 2-18years:58%. Overall, 78.5% of adults were infected with *H. pylori* and 36.6% of children were seropositive. The seropositivity increases with age; however, this might be an age-cohort effect. In addition, individual ages of children in the 2-18 year age group were not available, so the age at which infection was acquired could not be determined. This is important as the risk of cancer increases with early acquisition of *H. pylori* infection and acquiring the infection later in life could contribute to a lower cancer risk. Future work could determine the age at which the infection is acquired by studying the prevalence of *H. pylori* infection in this age group (2-18 years) in detail.

It has previously been shown that the early acquisition of *H. pylori* infection in childhood resulted in pangastritis in adulthood. This pattern of gastritis is usually associated with mucosal atrophy and intestinal metaplasia in adulthood both of which are precancerous conditions (Asaka et al., 1997, Atherton, 2006). Therefore, acquiring the infection at an early age is a recognised risk factor for the development of gastric cancer (Asaka et al., 1997, Atherton, 2006, Blaser et al., 2007). Additionally, antral predominant gastritis is usually associated with duodenal ulcer. In this project, histological changes in the anturm and the corpus were studied. There was no significant difference in *H. pylori* density, neutrophilic activity, intestinal metaplasia and mucosal atrophy between samples from antrum and corpus. Detailed scoring of gastric histopathology showed antral predominance of lymphocytic infiltration. This may, partially, help explain the low cancer risk in this population. However, due to the small sample size, it is impossible to conclude that the histology is representative of the overall population. To improve this, future work should focus on studying the hispotopathological changes in a larger representative sample.

The predominant *H. pylori* strains circulating in different geographic locations vary with regard to virulence genes (Atherton, 2006). An association of the *cagA*-positive, *vacA* s1 genotype with peptic ulcer disease (PUD) and gastric cancer has been reported in Western countries (Atherton, 2006). It was previously shown that strains with CagA proteins possessing greater numbers of EPIYA repeats increase phosphorylation of the protein, increase the extent of hummingbird phenotype formation, and are more likely to be associated with the development of gastric

cancer (Azuma et al., 2002, Higashi et al., 2002a, Higashi et al., 2005, Naito et al., 2006). Additionally, a recently described *vacA* i1 genotype has been shown to be associated with adenocarcinoma (Lu et al., 2005a). Furthermore, a strong association between *dupA* and duodenal ulcer has been reported (Lu et al., 2005a). Whether Iraqi strains possessed such genotypes was investigated and correlated with disease outcome and histological findings. The incidence of gastric cancer in Iraq appears lower than in Iran (GLOBOCAN, 2002). Therefore, virulence determinants of both Iraqi (Kurdish) and Iranian strains were studied and compared.

Strains from Iraq and Iran possessing *cagA* were found in 71% and 76% of the samples analysed, respectively. *cagA* presence was significantly associated with peptic ulcer disease incidence in Iraq but not in Iran. This might be because *cagA* is ubiquitous in Iran, and therefore not a good disease indicator. The presence of *cagA* alleles with more than 3 phosphorylation motifs was significantly higher amongst Iranian strains than those from Iraq. The presence of *cagA* with more phosphorylation motifs in Iran may help explain the higher cancer incidence rate in that country. There was no association between *vacA* signal and mid region and PUD in either population. A significant association between i1 genotype and incidence of GU was seen amongst samples from Iraq but not Iran. Recalling the correlation between *vacA* i region and gastric cancer, a relationship with GU appears logical because of the epidemiological association between gastric cancer and gastric ulcer. Furthermore, 33% and 38% of Iraqi and Iranian strains, respectively, were positive for *dupA*. Whilst a significant association between infection with *dupA*-positive strains and DU was observed for Iraqi patients, there

was no association among the Iranian subjects. The question of why *dupA* is associated with diseases in Iraq but not Iran could not be answered because our knowledge about this gene is limited.

This chapter has several limitations that can be addressed in future work. Perhaps most importantly we studied dyspeptic patients rather than a random community sample of *H. pylori* strains and this may have introduced bias. The reason we did this is that strain genotyping requires upper GI endoscopy, and so is difficult to perform on randomly selected asymptomatic individuals. We argue that the prevalence of virulent strains in the dyspeptic group without ulcer disease is likely to be similar to asymptomatic community members: the association between *H. pylori* infection and non-ulcer dyspepsia is controversial, and if present at all is weak, so any association with virulence factors is likely to be weaker still. Secondly, our study is not large. However, this is likely to hide true positive associations rather than produce false positives, and is most unlikely to have produced the multiple associations of virulence factors with disease that we have demonstrated. Thirdly, we have genotyped strains for virulence rather than performing virulence phenotyping. Many studies have shown good but imperfect associations between genotype and phenotype, but any error is likely to be in favour of designating a strain as virulent when in fact it is avirulent. For example, a seemingly virulent *cagA*+ strain may not be able to translocate CagA into epithelial cells (if it has a mutation elsewhere in the *cag* PAI which inactivates the type IV secretion system) and so may be avirulent. In contrast, a seemingly avirulent *cagA* negative strain will never be able to translocate CagA (as it lacks it) and so such a strain will never be virulent. Thus any errors from genotyping rather than

phenotyping are also likely to be conservative. Taken together these study deficiencies should encourage others to perform better studies to repeat and extend our investigation, but they do not negate our findings.

The immune responses against *H. pylori* in Kurdistan region, Iraq were studied. The levels of *FOXP3*, *IL-4*, *IL-10*, *IL-12* and *IFNγ* mRNA were found to be elevated in samples from infected patients compared to those from *H. pylori*-negative patients. However, the increase in Th2 cytokine levels was more marked than for Th1 cytokines. Th2 responses are associated with mild gastritis and low cancer risk (Atherton, 2006). Therefore, this might help explain the cancer low rate in Kurdistan region, Iraq. The association between female gender and a higher IL-4 (Th2) response may help to explain the lower gastric cancer incidence in women, although more studies are needed to investigate this relationship. A novel association between a high Th1 response and smoking was found suggesting that smoking may promote a pro-inflammatory Th1 response; such a response is known to induce precancerous gastric atrophy and metaplasia (Atherton, 2006). Unfortunately, no data about smoking status in Iraq was available. When using RT-PCR to investigate immune responses, the levels of cytokine mRNA are measured, but this does not always accurately reflect levels of cytokine protein expression and cannot show the cellular sources of cytokines. However, the levels of cytokines, especially IFNγ, are the crucial determinant for the disease process. For example, in a US study intestinal metaplasia developed in the stomach of mice transfused with IFNγ, even without *H. pylori* infection (Cui G, 2003). In addition, Robinson *et al.* used both RT-PCR and flow cytometry, which is regarded as the "gold-standard" method for determining numbers of Th1 and Th2 cells present. The results from

RT-PCR matched the results from flow cytometry (Robinson et al., 2008). Furthermore, whenever possible, we confirmed our results by studying the levels of IgG subclasses and their correlation with gastric cytokines. The association between smoking and Th responses is important. To take this work further, animal work might be helpful. However, current animal models of *H. pylori* infection are imperfect. In addition, burning cigarettes contains more than 400 chemical compounds. Testing which compound is responsible for promoting the Th-1 response would be extremely difficult. In addition, any human work in the future should consider using more samples from different regions of the world.

dupA is a very recently described *H. pylori* virulence factor (Lu et al., 2005a) and little is known about this gene. Lu *et al.* suggested that its possession is protective against cancer (Lu et al., 2005a) while Argent *et al.* showed a significant correlation between possess of the gene and gastric cancer (Argent et al., 2007). Based upon its sequence, *dupA* can be classified into two main categories: truncated *dupA* and *dupA* with a full open reading frame. It was found that disruption of *dupA* had no significant effect upon the level of interleukin-8 secretion from gastric epithelial cells. However, knockout of *dupA* with a complete open reading frame reduced IL-12, IFNγ , TNFα and IL-8 production by PBMCs significantly. This indicates that *dupA* might skew the immune response towards a Th-1 response. It was hypothesized that a functional *dupA* gene might be an important virulence factor in relation to the risk of clinically significant outcomes of *H. pylori* infection. *dupA* was associated with duodenal ulcer amongst Kurdish Iraqi subjects. However, these results raised question about which *dupA* polymorphism is common in this country. Hence, the presence of *dupA* is not enough and any study of the

correlation between *dupA* and clinical outcome should be accompanied by sequencing the gene. Raising antibodies against the DupA protein is recommended which would provide information on which form of this gene is expressed. In addition, determining which cells are reacting with *dupA*, using FACS, would be crucially important for the understanding of the disease development process and the role of immune response in this process. Finally, studying the signalling pathway would give an insight into understanding the mechanism of action of the DupA protein.

Lessons taken from this study and other studies (Atherton, 2006, Holcombe, 1992, Peek and Blaser, 2002, Singh and Ghoshal, 2006, Tham et al., 2001) show that *H. pylori* infection alone is not the only independent factor for the development of gastric cancer. Many studies conducted around the world (Archimandritis et al., 1993, Fukuda et al., 1995, Rudi et al., 1995, Talley et al., 1991b, Webb et al., 1996) imply that the role of *H. pylori* in the carcinogenesis is trivial in these countries. *H. pylori* virulence factors, smoking, gender, host genetic, environmental, and dietary factors play a major role in determining whether or not a person infected with *H. pylori* will develop gastric atrophy, intestinal metaplasia and gastric cancer. Each factor influences other factors in a way either increases or decreases the risk index. This has a crucial importance in preventive strategies of *H. pylori*-induced diseases. In the near future we may be able to conclude, for each *H. pylori*-infected subject, the risk of developing gastric cancer can be estimated using mathematical modelling of risk index. Based on this, he or she might be advised to take eradication therapy. If the index is low, patients might not be offered any antibiotic treatment. This is important economically and also might help reduce antibiotic

resistance. In addition, *H. pylori* infection has been suggested to protect from many diseases such as oesophageal carcinoma and allergies (Chen and Blaser, 2007, Sharma and Vakil, 2003, Wu et al., 2005). Thus, if patients could be shown to be of low risk of gastro-duodenal diseases, it may be beneficial not to treat *H. pylori*. Furthermore, if the index is high for all the population then rigorous development of a vaccination program on a population basis is justified, and all individuals with *H. pylori* related chronic gastritis should undergo eradication treatment.

7. References

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8. Appendix

A. Data showing endoscopic diagnosis, Smoking status, age, gender and cytokines mRNA expression in antral gastric biopsies

A.1 Table showing endoscopic diagnosis, smoking status, age, gender and cytokines mRNA expression in antral gastric biopsies

No.	Diagnosis	Smoking	Age	Sex	FOXP3	IL-10	IL-4	IL-12	IFN
1	GU	Yes	35	F	15.21	229.71	257.67	27.87	222.19
2	DU	Yes	40	M	3.86	2.54	0.23	20.84	64.97
3	DU	Yes	23	M	1.62	829.56	95.19	26.02	175.92
4	DU	Yes	18	M	1.02	1.14	0.67	11.04	111.30
5	DU	Yes	52	M	2.97	3.41	1.77	19.01	345.60
6	DU	No	28	M	1.17	3.01	2.54	2.20	2.64
7	GU	No	39	M	20.70	122.72	52.66	0.50	15.68
8	DU	No	28	F	9.24	162.11	67.67	0.40	6.37
9	NPUD	No	46	F	1.97	1.47	1.33	3.60	8.05
10	NPUD	No	80	F	1.00	45.65	25.08	0.10	3.41
11	NPUD	No	39	F	4.44	67.74	33.43	4.20	6.34
12	NPUD	No	70	F	3.10	306.28	6240.50	124.60	5.30
13	NPUD	No	18	F	3.67	443.69	181.76	11.20	8.60
14	NPUD	No	18	M	0.88	6.37	6.37	8.60	6.49
15	DU	Yes	18	F	4.72	1.19	1.19	97.20	12.55
16	NPUD	No	35	M	0.36	3.31	3.31	2.90	5.27
17	NPUD	Yes	20	F	12.40	381.04	145.64	1.70	2227.44
18	NPUD	No	23	F	0.63	198.82	76.56	33.50	1.14
19	NPUD	No	18	F	10.51	639.69	222.19	30.10	25.28
20	DU	Yes	28	M	7.28	101.42	38.08	1.00	2.54
21	NPUD	No	30	M	6.87	3.18	2.29	53.10	2.38
22	NPUD	No	28	F	24.05	319.94	229.71	2.50	10.00
23	NPUD	Yes	64	M	29.74	1.54	1.54	12.50	0.61
24	NPUD	Yes	23	F	22.75	539.49	486.16	24.40	14.14
25	NPUD	No	22	F	0.60	338.81	133.33	0.70	1.93
26	NPUD	No	22	F	0.93	10.62	7.62	82.70	3.90
27	GU	No	55	F	14.91	1.25	0.90	0.60	1.89
28	DU	Yes	35	M	0.32	1.54	1.02	30.11	115.86
29	DU	Yes	65	F	0.18	473.47	189.94	33.65	244.68
30	NPUD	Yes	45	F	1.88	7.62	0.67	31.61	146.02
31	GU	Yes	37	M	16.34	53.88	28.32	25.69	195.33
32	NPUD	Yes	76	M	1.46	68.78	32.35	175.30	13.78
33	DU	Yes	36	M	2.39	8.69	6.24	260.40	0.28
34	NPUD	Yes	70	F	5.18	486.60	172.04	0.10	13.19
35	GU	No	40	F	43.61	519.46	539.70	17.20	3.74

A.2 Table showing the differences in cytokines mRNA between *H. pylori*-positive and *H. pylori*-negative subjects

H. pylori-positive				
Hp+ FOXP3	Hp+ IL-10	Hp+ IL-4	Hp+ IL-12	Hp+ IFN
15.21	229.71	257.67	27.87	222.19
3.86	2.54	0.23	20.84	64.97
1.62	829.56	95.19	26.02	175.92
1.02	1.14	0.67	11.04	111.30
2.97	3.41	1.77	19.01	345.60
1.17	3.01	2.54	2.20	2.64
20.70	122.72	52.66	0.50	15.68
9.24	162.11	67.67	0.40	6.37
1.97	1.47	1.33	3.60	8.05
1.00	45.65	25.08	0.10	3.41
4.44	67.74	33.43	4.20	6.34
3.67	443.69	181.76	11.20	8.60
0.88	6.37	6.37	8.60	6.49
4.72	1.19	1.19	97.20	12.55
0.36	3.31	3.31	2.90	5.27
0.63	198.82	76.56	33.50	1.14
10.51	639.69	222.19	30.10	25.28
7.28	101.42	38.08	1.00	2.54
6.87	3.18	2.29	53.10	2.38
24.05	319.94	229.71	2.50	10.00
29.74	1.54	1.54	12.50	0.61
22.75	539.49	486.16	24.40	14.14
0.60	338.81	133.33	0.70	1.93
0.93	10.62	7.62	82.70	3.90
14.91	1.25	0.90	0.60	1.89
0.32	1.54	1.02	30.11	115.86
0.18	473.47	189.94	33.65	244.68
1.88	7.62	0.67	31.61	146.02
16.34	53.88	28.32	25.69	195.33
1.46	68.78	32.35	175.30	13.78
2.39	8.69	6.24	260.40	0.28
5.18	486.60	172.04	0.10	13.19
43.61	519.46	539.70	17.20	3.74

H. pylori-negative				
Hp- FOXP3	Hp- IL-10	Hp- IL-4	Hp- IL-12	Hp- IFN
0.52	2.43	0.98	2.27	1.22
1.26	0.89	0.36	2.52	0.68
0.52	0.09	0.00	0.88	1.02
1.25	0.03	0.17	0.07	1.75
1.27	0.05	0.02	0.86	1.16
0.68	1.36	0.00	1.76	1.10
0.89	0.02	0.00	0.07	1.33
1.00	0.00	0.04	1.45	1.45
1.07	0.00	0.00	0.96	1.21

A.3 Data analysis of cytokines mRNA secretion.

Variable	N	Mean	SE Mean	StDev	Median
Hp+ FOXP3	35	7.94	1.69	10.02	3.67
Hp+ IL-10	35	182.5	38.5	227.9	67.7
Hp+ IL-4	35	265	177	1048	32
Hp+ IL-12	35	33.63	9.30	55.03	17.20
Hp+ IFN	35	115.0	63.8	377.7	8.6
Hp- FOXP3	9	0.941	0.102	0.307	1.003
Hp- IL-10	9	0.541	0.287	0.862	0.046
Hp- IL-4	9	0.176	0.108	0.325	0.021
Hp- IL-12	9	1.205	0.291	0.872	0.962
Hp- IFN	9	1.2128	0.0981	0.2943	1.2123

Mann-Whitney Test Hp+ FOXP3, Hp- FOXP3

	N	Median
Hp+ FOXP3	35	3.666
Hp- FOXP3	9	1.003

Test is significant at 0.0062

Mann-Whitney Test Hp+ IL-10, Hp- IL-10

	N	Median
Hp+ IL-10	35	67.7
Hp- IL-10	9	0.0

Test is significant at 0.0000

Mann-Whitney Test Hp+ IL-4, Hp- IL-4

	N	Median
Hp+ IL-4	35	32.35
Hp- IL-4	9	0.02

Test is significant at 0.0000

Mann-Whitney Test Hp+ IL-12, Hp- IL-12

	N	Median
Hp+ IL-12	35	17.20
Hp- IL-12	9	0.96

Test ETA2 is significant at 0.0023

Mann-Whitney Test Hp+ IFN, Hp- IFN

	N	Median
Hp+ IFN	35	8.60
Hp- IFN	9	1.21

Test is significant at 0.0001

B. Data showing cytokines secretion from PBMC cells after co-culture with *H. pylori* strains.

B.1 Table showing data of IL-12 secretion from PBMC cells after co-culture with *H. pylori* strains and their Isogenic *dupA* mutant.

IL-12 Wild (AB31)	IL-12 Mutant (AB31)	IL-12 Wild (AB21)	IL-12 Mutant (AB21)	IL-12 Wild (93-67)	IL-12 Mutant (93-67)	IL-12 Wild (A101)	IL-12 Mutant (A101)
399.22	104.78	468.11	162.56	247.00	259.22	231.44	191.44
394.78	271.44	510.33	155.89	277.00	291.44	255.89	230.33
390.33	219.22	430.33	162.56	220.33	202.56	213.67	267.00
419.22	168.11	421.44	155.89	230.33	189.22	291.44	312.56
425.89	222.56	404.78	173.67	245.89	237.00	367.00	357.00

B.2 Data analysis of IL-12 secretion from PBMC cells after co-culture with *H. pylori* strains and their Isogenic *dupA* mutant.

Variable	N	Mean	SE Mean	StDev	Median
IL-12 Wild (AB31)	5	405.89	7.03	15.71	399.22
IL-12 Mutant (AB31)	5	197.2	28.3	63.3	219.2
IL-12 Wild (AB21)	5	447.0	18.9	42.3	430.3
IL-12 Mutant (AB21)	5	162.11	3.25	7.27	162.56
IL-12 Wild (93-67)	5	244.11	9.61	21.50	245.89
IL-12 Mutant (93-67)	5	235.9	18.6	41.6	237.0
IL-12 Wild (A101)	5	271.9	27.1	60.6	255.9
IL-12 Mutant (A101)	5	271.7	29.3	65.4	267.0

Paired T for IL-12 Wild (AB31) - IL-12 Mutant (AB31)

95% CI for mean difference: (125.7, 291.7)
T-Test of mean difference = 0 (vs not = 0): T-Value = 6.98 P-Value = 0.002

Paired T for IL-12 Wild (AB21) - IL-12 Mutant (AB21)

95% CI for mean difference: (226.6, 343.2)
T-Test of mean difference = 0 (vs not = 0): T-Value = 13.56 P-Value = 0.000

Paired T for IL-12 Wild (93-67) - IL-12 Mutant (93-67)

95% CI for mean difference: (-20.3, 36.7)
T-Test of mean difference = 0 (vs not = 0): T-Value = 0.80 P-Value = 0.468

Paired T for IL-12 Wild (A101) - IL-12 Mutant (A101)

95% CI for mean difference: (-46.4, 46.9)
T-Test of mean difference = 0 (vs not = 0): T-Value = 0.01 P-Value = 0.990

Paired T for IL-12 Wild (AB31) - IL-12 Wild (93-67)

95% CI for mean difference: (126.9, 196.7)
T-Test of mean difference = 0 (vs not = 0): T-Value = 12.87 P-Value = 0.000

Paired T for IL-12 Wild (AB21) - IL-12 Wild (A101)

95% CI for mean difference: (62.8, 287.4)
T-Test of mean difference = 0 (vs not = 0): T-Value = 4.33 P-Value = 0.012

B.3 Table showing data of IL-8, IFN-γ, TNF-α secretion from PBMC cells after co-culture with *H. pylori* strains and their Isogenic *dupA* mutant.

IL-8 Wild (AB31)	IL-8 Mutant (AB31)	IL-8 Wild (AB21)	IL-8 Mutant (AB21)	IFN Wild (AB31)	IFN Mutant (AB31)	IFN Wild (AB21)	IFN Mutant (AB21)	TNF Wild (AB31)	TNF Mutant (AB31)	TNF Wild (AB21)	TNF Mutant (AB21)
3004.74	2848.73	3725.57	3542.71	103.42	63.42	95.68	85.68	1354.78	1290.89	1779.46	1551.66
3064.71	2807.97	3772.71	3489.86	83.42	67.29	101.16	91.81	1398.11	1347.56	1763.86	1681.83
3079.95	2839.03	3788.43	3532.71	104.39	70.84	92.13	88.58	1400.33	1302.00	1807.25	1687.93
3172.41	2738.50	3898.43	3458.43	93.74	78.58	92.13	85.35	1407.56	1348.11	1833.02	1720.47
2894.27	2843.81	3597.00	3567.00	99.55	79.23	96.32	86.00	1360.89	1357.00	1828.27	1743.53

B.4 Data analysis of IL-8, IFN-γ, TNF-α secretion from PBMC cells after co-culture with *H. pylori* strains and their Isogenic *dupA* mutant.

Variable	N	Mean	SE Mean	StDev	Median
IL-8 Wild (AB31)	5	3043.2	45.9	102.7	3064.7
IL-8 Mutant (AB31)	5	2815.6	20.5	45.9	2839.0
IL-8 Wild (AB21)	5	3756.4	48.9	109.3	3772.7
IL-8 Mutant (AB21)	5	3518.1	19.5	43.5	3532.7
IFN Wild (AB31)	5	96.90	3.86	8.62	99.55
IFN Mutant (AB31)	5	71.87	3.10	6.94	70.84
IFN Wild (AB21)	5	95.48	1.67	3.72	95.68
IFN Mutant (AB21)	5	87.48	1.22	2.73	86.00
TNF Wild (AB31)	5	1384.3	11.0	24.5	1398.1
TNF Mutant (AB31)	5	1329.1	13.6	30.3	1347.6
TNF Wild (AB21)	5	1802.4	13.5	30.2	1807.3
TNF Mutant (AB21)	5	1677.1	33.3	74.4	1687.9

Paired T for IL-8 Wild (AB31) - IL-8 Mutant (AB31)

95% CI for mean difference: (51.9, 403.3)
T-Test of mean difference = 0 (vs not = 0): T-Value = 3.60 P-Value = 0.023

Paired T for IL-8 Wild (AB21) - IL-8 Mutant (AB21)

95% CI for mean difference: (52.6, 423.9)
T-Value = 3.56 P-Value = 0.024

Paired T for IFN Wild (AB31) - IFN Mutant (AB31)

95% CI for mean difference: (11.21, 38.85)
T-Test of mean difference = 0 (vs not = 0): T-Value = 5.03 P-Value = 0.007

Paired T for IFN Wild (AB21) - IFN Mutant (AB21)

95% CI for mean difference: (4.46, 11.54)
T-Test of mean difference = 0 (vs not = 0): T-Value = 6.27 P-Value = 0.003

Paired T for TNF Wild (AB31) - TNF Mutant (AB31)

95% CI for mean difference: (13.1, 97.4)
T-Test of mean difference = 0 (vs not = 0): T-Value = 3.64 P-Value = 0.022

Paired T for TNF Wild (AB21) - TNF Mutant (AB21)

95% CI for mean difference: (51.3, 199.3)
T-Test of mean difference = 0 (vs not = 0): T-Value = 4.70 P-Value = 0.009

B.5 Table showing data of IL-12 secretion from PBMC cells after co-culture with *H. pylori* strains and their Isogenic *dupA* and/or *cagE* mutant.

Wild (AB31)	*cagE*-mutant (AB31)	*dupA*-mutant (AB31)	double mutation (AB31)	Wild (93-67)	*cagE*-mutant (93-67)	*dupA*-mutant (93-67)	double mutation (93-67)
542.86	497.14	248.57	268.57	300.00	302.86	271.43	265.71
540.00	497.14	240.00	262.86	337.14	274.29	291.43	285.71
474.29	474.29	468.57	340.00	248.57	285.71	305.71	254.29
425.71	405.71	231.43	291.43	271.43	317.14	294.29	265.71
425.71	482.86	277.14	242.86	274.29	262.86	285.71	280.00

B.6 Data analysis of IL-12 secretion from PBMC cells after co-culture with *H. pylori* strains and their Isogenic *dupA* and/or *cagE* mutant.

Variable	N	Mean	SE Mean	StDev	Median
Wild (AB31)	5	481.7	25.9	58.0	474.3
cagE-mutant (AB31)	5	471.4	17.0	38.0	482.9
dupA-mutant (AB31)	5	293.1	44.5	99.6	248.6
double mutation (AB31)	5	281.1	16.6	37.2	268.6
Cells only	5	146.86	7.75	17.33	145.71
Wild (93-67)	5	286.3	15.1	33.8	274.3
cagE-mutant (93-67)	5	288.57	9.73	21.76	285.71
dupA-mutant (93-67)	5	289.71	5.61	12.55	291.43
double mutation (93-67)	5	270.29	5.61	12.55	265.71

Paired T for Wild (AB31) - *cagE*-mutant (AB31)

95% CI for mean difference: (-41.9, 62.5)
T-Test of mean difference = 0 (vs not = 0): T-Value = 0.55 P-Value = 0.613

Paired T for Wild (AB31) - *dupA*-mutant (AB31)

95% CI for mean difference: (38.2, 338.9)
T-Test of mean difference = 0 (vs not = 0): T-Value = 3.48 P-Value = 0.025

Paired T for Wild (AB31) - double mutation (AB31)

95% CI for mean difference: (111.9, 289.2)
T-Test of mean difference = 0 (vs not = 0): T-Value = 6.28 P-Value = 0.003

Paired T for Wild (93-67) - *cagE*-mutant (93-67)

95% CI for mean difference: (-56.2, 51.6)
T-Test of mean difference = 0 (vs not = 0): T-Value = -0.12 P-Value = 0.912

Paired T for Wild (93-67) - *dupA*-mutant (93-67)

95% CI for mean difference: (-54.5, 47.6)
T-Test of mean difference = 0 (vs not = 0): T-Value = -0.19 P-Value = 0.861

Paired T for Wild (93-67) - double mutation (93-67)

95% CI for mean difference: (-15.9, 47.9)
T-Test of mean difference = 0 (vs not = 0): T-Value = 1.39 P-Value = 0.236

B.7 Table showing data of IL-8 secretion from AGS cells after co-culture with *H. pylori* strains and their Isogenic *dupA* and/or *cagE* mutant.

Wild-type (AB31)	*dupA*-mutant (AB31)	*cagE*-mutant (AB31)	duoble mutant (AB31)	Wild-type (93-67)	*dupA*-mutant (93-67)	*cagE*-mutant (93-67)	duoble mutant (93-67)
4183.33	4250.00	950.00	1016.67	3783.33	3683.33	1183.33	983.33
4050.00	4183.33	883.33	1183.33	3916.67	3850.00	1016.67	1050.00
4953.33	4283.33	1150.00	1116.67	3750.00	4050.00	983.33	1016.67
4150.00	4016.67	1116.67	1150.00	4150.00	3950.00	816.67	983.33
3916.67	3783.33	1116.67	1316.67	3983.33	4550.00	883.33	1116.67
3950.00	4050.00	1116.67	1016.67	3916.67	3983.33	950.00	1016.67
6283.33	4016.67	916.67	1116.67	4083.33	3750.00	1050.00	1150.00
4316.67	4050.00	1083.33	1383.33	3983.33	4150.00	1016.67	1083.33

B.8 Data analysis of IL-8 secretion from AGS cells after co-culture with *H. pylori* strains and their Isogenic *dupA* and/or *cagE* mutant.

Variable	N	Mean	SE Mean	StDev	Median
Wild-type (AB31)	8	4475	283	800	4167
dupA-mutant (AB31)	8	4079.2	56.5	159.8	4050.0
cagE-mutant (AB31)	8	1041.7	37.7	106.5	1100.0
duoble mutant (AB31)	8	1162.5	46.3	130.9	1133.3
Wild-type (93-67)	8	3945.8	48.2	136.2	3950.0
dupA-mutant (93-67)	8	3995.8	95.9	271.4	3966.7
cagE-mutant (93-67)	8	987.5	39.1	110.5	1000.0
duoble mutant (93-67)	8	1050.0	21.8	61.7	1033.3

Paired T for Wild-type (AB31) - *dupA*-mutant (AB31)

95% CI for mean difference: (-272, 1064)
T-Test of mean difference = 0 (vs not = 0): T-Value = 1.40 P-Value = 0.203

Paired T for Wild-type (AB31) - *cagE*-mutant (AB31)

95% CI for mean difference: (2731, 4137)
T-Test of mean difference = 0 (vs not = 0): T-Value = 11.55 P-Value = 0.000

Paired T for Wild-type (AB31) - duoble mutant (AB31)

95% CI for mean difference: (2619, 4007)
T-Test of mean difference = 0 (vs not = 0): T-Value = 11.29 P-Value = 0.000

Paired T for Wild-type (93-67) - *dupA*-mutant (93-67)

95% CI for mean difference: (-292, 192)
T-Test of mean difference = 0 (vs not = 0): T-Value = -0.49 P-Value = 0.641

Paired T for Wild-type (93-67) - *cagE*-mutant (93-67)

95% CI for mean difference: (2775.4, 3141.2)
T-Test of mean difference = 0 (vs not = 0): T-Value = 38.25 P-Value = 0.000

Paired T for Wild-type (93-67) - duoble mutant (93-67)

95% CI for mean difference: (2790.0, 3001.6)
T-Test of mean difference = 0 (vs not = 0): T-Value = 64.73 P-Value = 0.000

C. Publications

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Differences in Virulence Markers between *Helicobacter pylori* Strains from Iraq and Those from Iran: Potential Importance of Regional Differences in *H. pylori*-Associated Disease▽

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***Helicobacter pylori* causes peptic ulceration and gastric adenocarcinoma; the latter is common in Iran but not in Iraq. We hypothesized that more virulent *H. pylori* strains may be found in Iran than in Iraq and so compared established and newly described virulence factors in strains from these countries. We studied 59 unselected dyspeptic patients from Iran and 49 from Iraq. *cagA* was found in similar proportions of strains from both countries (76% in Iran versus 71% in Iraq) and was significantly associated with peptic ulcer disease in Iraq ($P \leq 0.01$) but not in Iran. *cagA* alleles encoding four or more tyrosine phosphorylation motifs were found in 12% of the Iranian strains but none of the Iraqi strains ($P = 0.02$). There were no significant differences in the *vacA* signal-, middle-, or intermediate-region types between Iranian and Iraqi strains. Among the strains from Iran, *vacA* genotypes showed no specific peptic ulcer associations, but among the strains from Iraq, *vacA* i1 strains were associated with gastric ulcer ($P \leq 0.02$), mimicking their previously demonstrated association with gastric cancer in Iran. *dupA* was found in similar proportions of Iranian and Iraqi strains (38% and 32%, respectively) and was associated with peptic ulceration in Iraqi patients ($P \leq 0.01$) but not Iranian patients. *H. pylori* strains from Iraq and Iran possess virulence factors similar to those in Western countries. The presence of *cagA* with more phosphorylation motifs in Iranian strains may contribute to the higher incidence of gastric cancer. However, the association between strain virulence markers and disease in Iraq but not Iran suggests that other host and environmental factors may be more important in the disease-prone Iranian population.**

Helicobacter pylori is a spiral-shaped, gram-negative bacillus which causes gastritis and peptic ulceration (18, 19, 36). Its treatment has become pivotal in the management of peptic ulcer disease (PUD). *H. pylori* infection is also an important risk factor for gastric adenocarcinoma, the second most important cause of cancer deaths worldwide. Gastric cancer is thought to have a multifactorial etiology; and bacterial strain type, host genotype, and environmental conditions are all thought to be factors contributing to gastric cancer (22). Despite the geographical proximity of Iraq and Iran, the incidence of gastric cancer differs hugely between these countries; in Iran it ranges from 38 to 69 cases/10^5 population (10, 21, 26, 27, 38), whereas in Iraq the incidence is 5 cases/10^5 population (10). We hypothesized that this difference may be due to differences in the virulence of the circulating *H. pylori* strains, and so we set out to type strains from these countries for their virulence. We considered both well-established and more recently described virulence determinants.

Many strains of *H. pylori* produce the CagA protein, encoded by the *cagA* gene within the *cag* pathogenicity island (PAI). *H. pylori* strains possessing *cagA* are associated with a significantly increased risk for the development of atrophic gastritis, PUD, and gastric cancer (24, 32). The *cag* PAI encodes a type IV secretion system that facilitates the translocation of CagA into the host epithelial cytosol, where it becomes phosphorylated with tyrosine at specific phosphorylation motifs by the Src family of kinases (29, 30). Phosphorylated CagA forms a physical complex with SHP-2 phosphatase and stimulates cell signaling pathways, cytoskeletal changes, and abnormal cell proliferation (33). On the basis of the amino acid sequence of the SHP-2 binding site, CagA proteins can be subcategorized into Western and East Asian types. Both have type A and B phosphorylation motifs (usually one of each), but the Western types have additional C motifs (1–3) and the East Asian type has no C motifs but a D motif. The East Asian type CagA possesses stronger SHP-2 binding and transforming activities than the Western type CagA (11). The Western type CagA has a variable number of type C phosphorylation motifs, and the extent of cytoskeletal changes induced by CagA is

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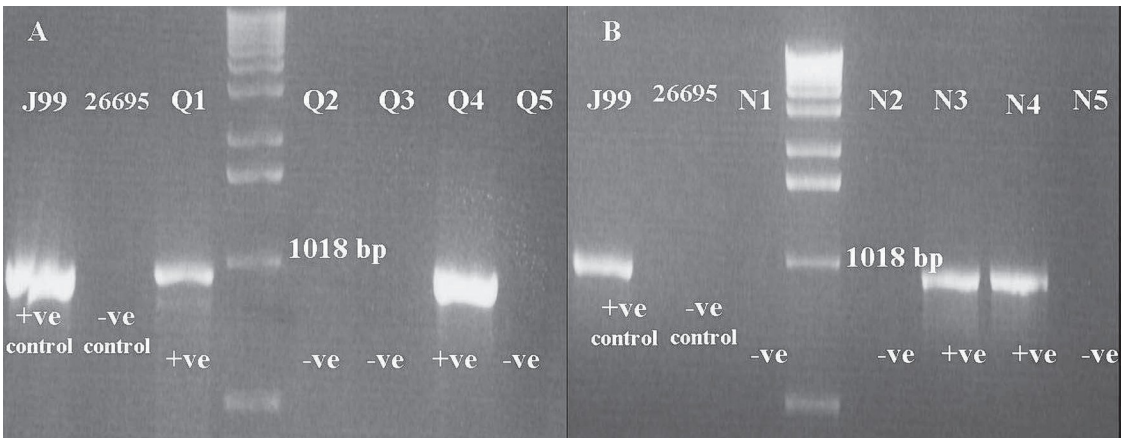

FIG. 1. Characterization of Iraqi (A) and Iranian (B) strains for *dupA* by PCR. Image shows the results of PCR typing of *dupA* with primers DupAF113 and DupAR1083. +ve, positive; −ve, negative.

dependent on this. Strains possessing CagA with greater numbers of type C phosphorylation motifs are more closely associated with gastric carcinogenesis (9). Thus, determination of the degree of CagA phosphorylation or the number of phosphorylation motifs appears to be more important than detection of *cagA* alone (4, 5).

The vacuolating cytotoxin (VacA) is a well-established *H. pylori* virulence factor which has multiple effects, including vacuolization of cultured epithelial cells, the induction of apoptosis, increases in the permeability of epithelial monolayers, the formation of pores in cells, and suppression of immune cell function (6, 13). The *vacA* gene is polymorphic within its signal, intermediate, and middle regions. For the signal region, two distinct allelic sequences, s1 and s2, have been recognized. For the middle region, alleles can be categorized into two classes, m1 or m2. The *vacA* genotype is associated with in vitro cytotoxin activity (with s1 having greater cytotoxin activity than s2 and m1 having greater cytotoxin activity than m2) (8, 20, 34, 35). Rhead et al. have recently described a novel determinant of VacA toxicity, the intermediate or i region (23). They showed that two allelic variants of this region, i1 and i2, exist. Furthermore, they showed that only s1/m2 strains varied in their i types; s1/m1 and s2/m2 strains were exclusively i1 and i2, respectively. This novel region determines the vacuolating activity among these s1/m2 strains. Most importantly, a significant correlation was found between the i1 type of *vacA* and gastric cancer in Iran (23).

The duodenal ulcer (DU)-promoting gene A (*dupA*) is a recently described virulence factor which comprises both jhp0917 and jhp0918 (16). Lu et al. found a significant relationship between *dupA* and DU, and the presence of *dupA* was related to neutrophil infiltration and a high level of interleukin-8 production by epithelial cells. Surprisingly, possession of this gene appeared to be protective against gastric adenocarcinoma (16).

The object of this study was to type the virulence of unselected strains from dyspeptic patients in Iran and Iraq. We aimed to compare the virulence of strains from these neighboring countries, which have very different incidences of gastric cancer, and to assess the association of virulence markers with PUD in each country.

MATERIALS AND METHODS

Patient-derived samples. Gastric biopsy specimens were obtained from 49 and 59 unselected *H. pylori*-positive patients from Iraq and Iran, respectively, undergoing routine upper gastrointestinal endoscopy for investigation for dyspepsia. The mean age ± standard deviation of the Iraqi patients was 35 ± 17 years, and that of the Iranian patients was 40 ± 14 years. All Iraqi patients were from the five districts of city of Dohuk. The majority (48/59 [81%]) of Iranian patients were from the city of Tehran; other patients were referred from different regions in Iran. Endoscopic diagnoses were as follows: for DU, 15 in Iraq and 8 in Iran; for gastric ulcer (GU), 5 in Iraq and 9 in Iran; for no ulcer disease, 29 in Iraq and 42 in Iran. During gastroscopy, biopsy samples were taken and either placed in 1 ml of Iso-Sensitest broth (Oxoid, Basingstoke, United Kingdom) containing 15% (vol/vol) glycerol and stored in liquid nitrogen or cultured immediately for *H. pylori*. In some cases, following prolonged storage and shipment to the United Kingdom, reculture was not possible. In these cases, DNA was extracted directly from the biopsy specimens and used for PCR-based *H. pylori* typing.

The study protocol was approved by the ethics and research committees of the individual hospitals, and all patients gave informed consent to participation in the study.

Culture. Each biopsy specimen was spread onto horse blood or Dent agar plates and then incubated under microaerobic conditions generated with a CampyPak system (Becton Dickinson, Baltimore, MD) in an anaerobic jar at 37°C for 2 to 4 days. The organisms were identified as *H. pylori* by colony morphology, Gram stain, and urease activity. Cultures were harvested as sweeps rather than single colonies and were stored in Iso-Sensitest broth containing 15% (vol/vol) glycerol at −80°C.

Genotyping of *H. pylori*. PCR-based typing of the *H. pylori* isolates was performed with DNA extracted from bacteria or directly from the biopsy specimens. PCR amplification of *cagA* used previously described primers cag2 and cag4 (25) to amplify the 3′ variable region. PCR amplification of the *cag* PAI empty site was performed as described previously (1). In the empty-site PCR, primers anneal to sequences adjacent to the *cag* PAI insertion site in the genome and allow amplification only of a DNA fragment of the expected size in the absence of a complete or partial *cag* PAI at this locus. Genotyping of the *vacA* signal, intermediate, and middle regions was performed as described previously (7, 8, 23). *dupA* was amplified with primer pairs DupAF113-DupAR1083 and DupAF1202-DupA918R, as described previously (3) (Fig. 1). Determination of the number of CagA phosphorylation motifs and the types of motifs was carried out by using the forward primer cag2 and the reverse primers cagA-P1C, cagA-P2CG and cagA-P2TA (the B motif is polymorphic, and reverse primers cagA-P2CG and cagA-P2TA are designed to recognize all types described to date), and cagA-P3E, as described previously (5) (Fig. 2). Five microliters of the PCR products was electrophoresed in 1.5% (wt/vol) agarose gels for 40 min at 80 V in TAE (Tris-acetate-EDTA) buffer. All gels were stained with ethidium bromide (1 mg/liter) and photographed under UV light. A 1-kb DNA ladder (Gibco, Paisley, United Kingdom) was used as a size marker in all gels. Strains with previously determined genotypes were used as positive controls.

Data analysis. Statistical analysis of the data was performed by using logistic regression, the chi-square test, and Fisher's exact test, with significance set at a

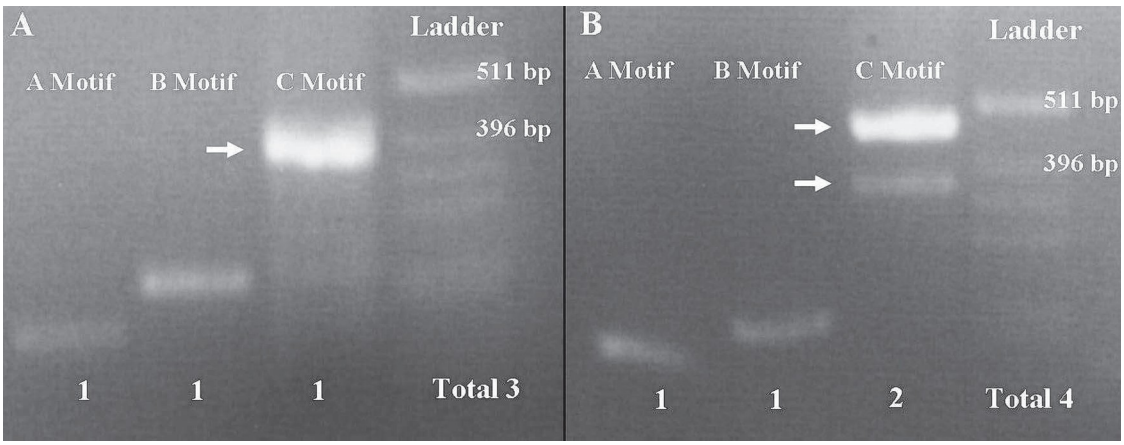

FIG. 2. Characterization of one Iraqi strain (A) and one Iranian strain (B) for *cagA* variable-region tyrosine phosphorylation motifs. Genomic DNA samples from *H. pylori* strains were used to PCR amplify the *cagA* variable-region EPIYA motifs by using forward primer cag2 and reverse primer cagA-P1C (A motif), reverse primers cagA-P2CG and cagA-P2TA (B motif), or reverse primer cagA-P3E (C motif). No Iraqi strain was found to have more than three phosphorylation motifs.

P value of <0.05. Genotypes with mixed status for *vacA* were excluded from the calculations of association.

RESULTS

Prevalence of *cagA*$^+$ strains among dyspeptic Iranian and Iraqi populations. First, we assessed whether the prevalence of *cagA*-positive (*cagA*$^+$) strains was similar or different between the Iraqi and the Iranian populations. *cagA*$^+$ strains were present in 76% (45/59) and 71% (35/49) of the *H. pylori* strains from unselected Iranian and Iraqi patients with dyspepsia, respectively (Table 1). To exclude bias from disease association, we also compared subgroups of patients without peptic ulceration: *cagA* was found in a higher proportion of Iranian strains than Iraqi strains (76% and 55%, respectively), although this did not quite achieve formal statistical significance ($P = 0.06$). In both countries, all *cagA*$^+$ strains also typed positive for *cagE*. Among 14 *cagA*-negative Iraqi strains, 7 were *cag* PAI empty-site positive (implying that the whole *cag* PAI was absent) and 7 were empty-site negative (implying that there was still a partial *cag* PAI at this locus). Among 14 *cagA*-negative Iranian strains, 9 were *cag* PAI empty-site negative. Thus, overall, the *cag* PAI appeared to be incompletely deleted in 16 strains.

No significant association was found between *cagA* status and clinical outcome for the Iranian patients, but a significant correlation was found between *cagA* and PUDs ($P \leq 0.01$; odds ratio, 16.4) in Iraqi patients (Table 1). When DU and GU were considered separately for the Iraqi population, 14/15 (93%) patients with DU had *cagA*$^+$ strains, whereas 16/29 (55%) patients with no ulcer ($P < 0.02$) had *cagA*$^+$ strains. All Iraqi GU patients had *cagA*$^+$ strains (*P* was not significant compared with the results for patients with no ulcer, perhaps due to the low number of GU patients).

CagA phosphorylation motif numbers. Second, we turned our attention to *cagA* polymorphisms and, in particular, the number of CagA phosphorylation motifs, which we assessed using our recently described PCR-based typing system (5). Among the *cagA*$^+$ strains, 12% (7/49) of the strains from Iran carried a *cagA* variable region of >550 bp (when the region was amplified with primers cag2 and cag4), indicating the presence of more than three CagA phosphorylation motifs. This was a significantly higher proportion than that found in the strains from Iraq, where all strains possessed *cagA* with a variable-region size of 550 bp, indicating the presence of CagA with three phosphorylation motifs ($P = 0.02$) (Table 1). In the analysis confined to patients without ulcers, 22% (7/32) of the *cagA*$^+$ Iranian strains had more than three phosphorylation sites, whereas none of the Iraqi strains did. Previous studies with other populations have linked multiple CagA phosphorylation motif numbers with an increased risk of cancer but not

TABLE 1. *cagA* status, *cagA* phosphorylation motif number, and *dupA* status among *H. pylori* strains from unselected Iranian and Iraqi patients with dyspepsia

Country	% of strains positive for:								
	cagA			More than three *cagA* phosphorylation motifs			*dupA*		
	PUD	NPUD[a]	Total	PUD	NPUD	Total	PUD	NPUD	Total
Iraq	95 (19/20)[b,c]	55 (16/29)	71 (35/49)	0 (0/20)	0 (0/29)	0 (0/49)	55 (11/20)[b]	17 (5/29)	32 (6/49)
Iran	76 (13/17)	76 (32/42)	76 (45/59)	0 (0/17)	17 (7/42)	12 (7/59)[d]	35 (6/17)	40 (17/42)	39 (23/59)

[a] NPUD, no PUD.
[b] $P < 0.05$ for comparison of patients with PUD and patients without PUD.
[c] Values in parentheses indicate the number of strains positive/total number of strains tested.
[d] The presence of *cagA* alleles with more than three phosphorylation motifs was significantly greater among Iranian strains than among Iraqi strains ($P = 0.02$).

TABLE 2. Distribution of *vacA* allelic types among *H. pylori* strains isolated from unselected dyspeptic patients from Iraq and Iran

Country	No. of patients positive for the following allelic type/total no. (%):					
	s1/i1/m1	s1/i1/m2	s1/i2/m1	s1/i2/m2	s2/i2/m2	Mixed
Iraq	8/49 (16.3)	2/49 (4.1)	1/49 (2.0)	20/49 (40.8)	4/49 (8.2)	14/49 (28.5)
Iran	15/59 (25.4)	4/59 (6.7)	1/59 (1.7)	16/59 (27.1)	16/59 (27.1)	7/59 (11.9)

with an increased risk of ulcer (9, 15): in agreement with this, no strains with more than three phosphorylation motifs were found in the ulcer group from Iran (Table 1).

***vacA* polymorphism.** We then turned to *vacA* polymorphisms in Iranian and Iraqi strains, examining both established s and m genotypes, and also the recently described polymorphic i-region type. Since individual *H. pylori* isolates possess only a single copy of *vacA*, the presence of more than one *vacA* s, i, or m genotype in a DNA sample indicates colonization by two or more strains with different *vacA* genotypes (8). Among the Iraqi isolates, a single *vacA* signal region was observed in all samples, but 8/49 (16%) of the specimens examined possessed both middle-region types, and 9/49 (18%) possessed both i-region genotypes. Among the Iranian samples, all isolates possessed a single signal-region type, but 2/59 (3%) carried both m-region types and 7/59 (12%) possessed both i-region types. There was no difference in the prevalence of strains with different *vacA* genotypes among the unselected dyspeptic populations from Iran and Iraq, whether strains with multiple genotypes were excluded (planned analysis; Table 2) or classified as the more pathogenic or the less pathogenic type (exploratory analyses).

Next, we examined associations between *vacA* allelic variation and peptic ulceration within the Iranian and Iraqi populations. For the Iranian strains, no significant associations were found. For the Iraqi strains, no significant association was found for duodenal ulceration, but 80% (4/5) of the strains isolated from GU patients were of the *vacA* i1 genotype, which was significantly greater than the 13% (4/29) of the strains from patients without ulcers ($P < 0.02$). Although this subgroup analysis is exploratory, it is interesting, given the described association between *vacA* i1 genotype and gastric cancer and the similarities in epidemiology and pathogenesis between GU and gastric cancer. Associations were not seen between gastric ulceration and the *vacA* s and m types, again supporting the recent finding that the *vacA* i type is a better marker of strain virulence (23).

***dupA* status.** Third, we examined strains for the recently described putative virulence gene *dupA*. Similar proportions of Iranian and Iraqi strains possessed *dupA* (Table 1). Among the Iranian patients, we found no association between *dupA* and the clinical outcome. However, among the Iraqi patients, 55% (11/20) of the peptic ulcer patients carried *dupA*$^+$ strains, significantly more than the 17% (5/29) of the patients without ulcers who carried *dupA*$^+$ strains ($P < 0.01$; odds ratio, 6.2) (Table 1). When we looked at DU and GU separately, 60% (9/15) of the *H. pylor*i isolates from DU patients were *dupA*$^+$ ($P < 0.01$ compared with the results for patients without ulcers) and 40% (2/5) of the *H. pylori* isolates from GU patients were *dupA*$^+$ (*P* was not significant compared with the results for the patients without ulcers, but note the small number of GU patients).

Associations between virulence factors, particularly for *cagA* phosphorylation motif number. Next, we assessed associations between virulence factors in strains from Iran and Iraq. As in virtually all strain populations worldwide, we found that *cagA*$^+$ strains were more likely to be *vacA* s1 than s2: in Iran, 37/45 (82%) *cagA*$^+$ strains were *vacA* s1, whereas 5/14 (36%) of the *cagA*-negative strains were *vacA* s1 ($P < 0.005$); in Iraq, all *cagA*$^+$ strains typed s1, whereas 10/14 (71%) of the *cagA*-negative strains typed s1 ($P < 0.005$). No significant associations were found between *cagA* status and other *vacA* polymorphisms or between *cagA* status and *dupA* status. As strains with a larger *cagA* are thought to be more pathogenic than those with a smaller *cagA*, we examined the association between the size of *cagA* and other virulence factors among Iranian strains. This analysis was not possible for Iraqi strains, as they all had the same number ($n = 3$) of CagA phosphorylation motifs. Seven of 45 (15%) *cagA*$^+$ Iranian strains carried a larger *cagA* (with more than three phosphorylation motifs). In the association analysis with *vacA* genotypes, we excluded patients with mixed genotypes. The small numbers of strains studied meant that most associations were not statistically significant, but for the *vacA* m region, 6/7 (86%) strains with more than three phosphorylation motifs were type m1, significantly more than the 10/37 (27%) strains with only three phosphorylation motifs ($P = 0.01$). Lastly, we examined the association between the size of *cagA* and *dupA* status: 6/7 (86%) strains with more than three phosphorylation motifs were *dupA*$^+$, significantly more than the 14/38 (36%) strain with only three phosphorylation motifs ($P = 0.03$).

DISCUSSION

The study of *H. pylori* virulence factors in populations is important, as they contribute to disease risk. For example, in Japan, where gastric cancer is common, more than 90% of *H. pylori* strains are *cagA* positive (17). The gastric cancer rate in Iraq is lower than that in Iran; we hypothesized that differences in the virulence factors of the *H. pylori* strains between these two countries may partially explain this difference. We found no difference in the prevalence of *cagA*$^+$ strains between unselected dyspeptic populations from these countries, although among patients without ulcers, *cagA*$^+$ strains were 21% more prevalent in Iran ($P = 0.06$). Furthermore, Iranian patients with *cagA*$^+$ strains were more likely to have the more pathogenic forms of *cagA* encoding four or more tyrosine phosphorylation sites, and among patients without ulcers, this difference was 22%. Taking these results together, this represents a considerable difference in potential *cagA*-associated pathogenicity

which could contribute to the differences in gastric cancer rates seen between these populations: both *cagA* status and the number of *cagA* phosphorylation motifs have been linked with cancer prevalence in a number of populations (4, 11). However, we found no significant differences between Iranian and Iraqi populations in *vacA* types and, in particular, in the i-region type, which has recently been linked with gastric cancer risk in Iran (23). Also, we found no difference in *dupA* status, which we studied because *dupA* has been reported to have a negative association with gastric cancer (16), although recent data from us dispute this (3).

In the present report, we have shown that 71% and 76% of the *H. pylori* strains isolated from Iraqi and Iranian samples, respectively, were *cagA*$^+$. This value is closer to the values for Western countries and Turkey than to the values for East or Southeast Asia (2, 14, 28, 31). Our strains had the Western type of CagA and the Western types of *vacA*. Thus, it appears that the high cancer rate in Iran is not due to the presence of strains of the East Asian type in that country.

We looked within the Iranian and Iraqi populations for associations between virulence factors and PUD. Among the Iraqi strains but not the Iranian strains, we observed an association between *cagA*$^+$ status and PUD. Reports from a neighboring country, Turkey, have shown results similar to those from Iraq (28). No Iraqi strains had *cagA* with more than three phosphorylation motifs, so we could not perform an examination for disease associations. The situation in Iran was interesting: no strains with more than three phosphorylation motifs were found in patients with peptic ulcer. This may imply that the presence of more than three phosphorylation motifs is protective against ulcers rather than being a specific predisposition to cancer, as reported previously (4, 11). For *vacA* polymorphisms, we found no association between the *vacA* i region and the clinical outcome for the Iranian samples. However, for the Iraqi specimens, a novel association was found between the presence of *vacA* i1 strains and gastric ulcer. This is not unexpected, as GU and gastric cancer are epidemiologically similar. However, our results need confirmation by the performance of studies with other populations, as only a small number of GU patients were enrolled in this study. For *dupA*, a significant link with PUD was present in the Iraqi population, but no association was found in Iranians. Thus, overall, we showed that the Iraqi population was similar to Western populations in terms of the association of many virulence factors with ulcer disease. In contrast, these associations were not seen in the Iranian population. This may imply that factors other than bacterial virulence are the most important for ulcer risk in Iran.

Many previous reports have shown a clustering of active virulence factors within *H. pylori* strains, for example, associations between *cagA*$^+$ status and the *vacA* s1 genotype (37). In agreement with the findings presented in those reports, we found a significant correlation between *cagA*$^+$ status and the presence of the *vacA* s1 genotype in Iran and Iraq. In addition, we showed a significant association between the presence of a greater number of *cagA* phosphorylation motifs and the presence of both the *vacA* m1 genotype and *dupA*$^+$ status. This further supports the concept of the clustering of virulence factors, such that the majority of *H. pylori* strains possess either many or a few, and the fact that it is favorable for *H. pylori* to be either strongly pathogenic or nonpathogenic.

Our study has several limitations. First, and perhaps most importantly, we studied *H. pylori* strains from dyspeptic patients rather than a random community sample of *H. pylori* strains, and this may have introduced bias. The reason that we did this is that strain genotyping requires upper gastrointestinal endoscopy, which is difficult to perform with randomly selected asymptomatic individuals. We argue that the prevalence of virulent strains in the dyspeptic group without ulcer disease is likely to be similar to that in asymptomatic community members: the association between *H. pylori* infection and nonulcer dyspepsia is controversial, and if such an association is present at all, it is weak; so any association with virulence factors is likely to be weaker still. Second, our study was not large. However, this is more likely to hide true-positive associations rather than to produce false-positive results and is most unlikely to have produced the multiple associations of virulence factors with disease that we have demonstrated. Third, we genotyped strains for virulence rather than perform virulence phenotyping. Many studies have shown good but imperfect associations between the genotype and the phenotype of a strain, but any error is likely to be in favor of designating a strain as virulent when, in fact, it is avirulent. For example, a seemingly virulent *cagA*$^+$ strain may not be able to translocate CagA into epithelial cells (if it has a mutation elsewhere in the *cag* PAI which inactivates the type IV secretion system) and so may be avirulent. In contrast, a seemingly avirulent *cagA*-negative strain will never be able to translocate CagA (as it lacks it), and so such a strain will never be virulent. Thus, any errors from genotyping rather than errors from phenotyping are also likely to be conservative. Taken together these study deficiencies should encourage others to perform better studies to repeat and extend our investigation, but they do not negate our findings.

To summarize, the virulence factors of both Iraqi and Iranian *H. pylori* strains appear to be more closely related to strains from Western countries than to strains from Asian countries. Iranian strains appear to be more virulent, but the difference appears to be unlikely completely to explain the difference in disease prevalence between these countries. This suggests that unidentified strain, host, and environmental factors may contribute to these differences. In the absence of an East Asian type of *cagA* and almost universally virulent strains (as are found in Japan and parts of China), the very high gastric cancer rate in Iran remains largely unexplained. Similarly, the cancer rates in Iraq appear to be lower than those that would be expected from the circulating *H. pylori* strain types, an enigma similar to that reported (controversially) in Africa (12).

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LETTER TO EDITOR

A study of Age-Specific *Helicobacter pylori* Seropositivity Rates in Iraq

Dear Editor

Helicobacter pylori is regarded as the most important risk factor for peptic ulcer disease and gastric cancer [1]. In Iraq, gastric cancer is rare [2]. Despite the geographic proximity of Iraq, Turkey, and Iran the incidence of gastric cancer differs hugely among these countries; $\leq 5/10^5$, 8.9 to $14.1/10^5$, and 38 to $69/10^5$, respectively [2]. One possible explanation for these differences would be similar differences in prevalence of *H. pylori* infection. However, data from Turkey and Iran do not support this idea: in eastern Turkey, 64% of the population were found to be seropositive for *H. pylori* [3], whereas in Iran, *H. pylori* seroprevalence was only 33% [4]. In another study from Iran, *H. pylori* seropositivity was 48% in Ardebil province and 31% in Yazd province [5]. Gastric cancer is more common in Ardebil than Yazd; in these two areas differences in *H. pylori* prevalence may be contributing to the difference. The prevalence of *H. pylori* in Iraq is unknown. Thus, we now aimed to investigate whether *H. pylori* infection was uncommon and whether it occurred late in life in Iraq.

Materials and Methods

Patient-Derived Samples: Serum samples from 283 subjects including 120 children and 163 adults admitted to the surgical and pediatric wards for conditions other than gastrointestinal disorders were used in the study. The study protocol was approved by the Ethics and Research Committees of the hospital, and all patients gave informed consent to the study.

Antigen Preparation for ELISA: A lysate of five Iraqi *H. pylori* stains (all of which were *cagA*-positive) was prepared by sonicating a suspension of a pool of the five Iraqi strains in PBS. This procedure was performed on ice using six cycles of 30 seconds, followed by 30 seconds cooling. The protein concentration was determined using a modified Lowry protein assay kit (Bio-Rad, Hercules, CA, USA).

Serum IgG ELISA: Wells of a 96-well ELISA plate (NUNC maxisorp, Roskilde, Denmark) were coated with 50 µL *H. pylori* sonicate antigen diluted to 3 ng/mL in 0.1 mol/L carbonate/bicarbonate coating buffer (pH 9.6). The plates were then incubated overnight at 4 °C. One hundred microlitres of blocking solution (3% bovine serum albumin in PBS-Tween) was added to each well. Sera were tested in a twofold dilution series, including replicate wells of a 1/50 dilution of a pool of negative control sera from four uninfected donors on every plate. Fifty microlitres of diluted (1/50,000), antihuman IgG-HRP (Sigma A8667) was added before development using tetra-methylbenzidine substrate (eBioscience, San Diego, CA, USA). The optical density of the samples was measured using a microplate reader (Labsystems iEMS Reader MF, Helsinki, Finland) at a wavelength of 450 nm with a reference wavelength of 650 nm. Then, curves of dilution against mean OD for each sample were plotted. Statistics: Data were analyzed using the Minitab 15 software program. The chi-squared test was performed to study the difference in positivity rate between age groups. Differences were considered statistically significant at $p < .05$.

Results and Discussion

The prevalence of *H. pylori* in children of different ages varied (age and percentage: 6 months and 0%; 6–24 months and 27%; 2–18 years and 58%) with an overall prevalence of *H. pylori* in children of 36% (44 of 120). Seventy-eight percent (128 of 163) of adults were infected with *H. pylori*, a significantly higher proportion than children ($p < .0001$). The prevalence of *H. pylori* increased markedly with age through childhood with the maximum colonization (81.5%) occurring in adults in the 18- to 40-year-old-group (Fig. 1). Among adults, prevalence was similar between age groups (Fig. 1). Sixty-three percent (97 of 153) of males were infected with *H. pylori*, while 58% (76 of 130) of females were *H. pylori*-seropositive (p = not significant).

Although *H. pylori* organisms have been isolated worldwide, they are more frequently recovered from patients in developing countries [1]. Our cross-sectional population-based serosurvey demonstrated that the prevalence of *H. pylori* infection among hospitalized patients in Dohuk City, Iraq, was 61%. The seropositivity was not related to gender. In agreement with other studies [6,7], a significant increase in the seroprevalence of *H. pylori* infection among asymptomatic children by age was found. The prevalence of *H. pylori* infection in Iraq is higher than reported in Iran (33%) [4], where the gastric cancer rate is very high and lower than Turkey (64.4%), where the gastric cancer rate

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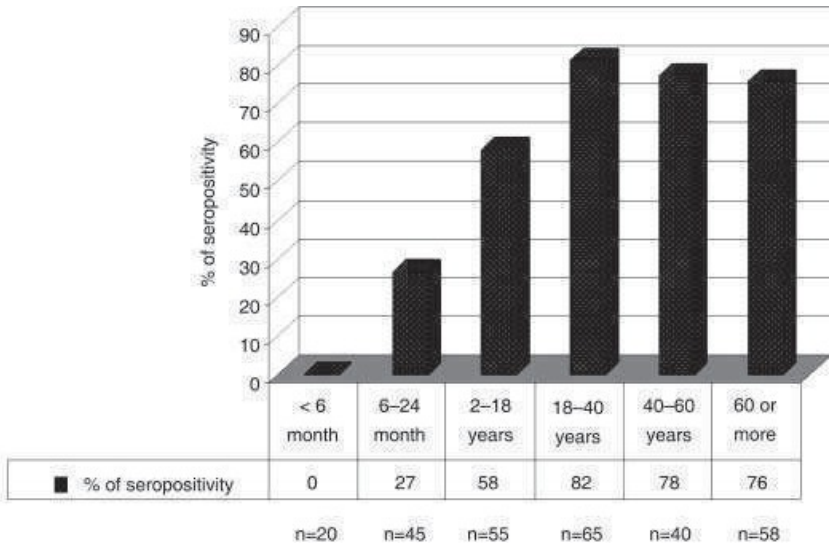

Figure 1 The seropositivity of *Helicobacter pylori* infection in relation to age.

is intermediate [2]. Thus, there is not a simple positive association between *H. pylori* and gastric cancer rate in these three countries, and the lower gastric cancer rate in Iraq is not due to low prevalence of *H. pylori*. According to our IgG-based ELISA, none of the children younger than 6 months were found to be seropositive. These might be false negative results as the immune system in children of this age is not fully mature. However, 27% of the subjects aged 6 months to 2 years were seropositive in our assay and so appear to have acquired the infection. Unfortunately, we do not have individual ages of children in the 2–18 years age group, so we cannot comment further on the most common period of infections between these ages. However, it is clear from our results that at least quarter of children are infected very early in life, and most infections in Iraq occur during childhood. Thus, our study suggests that the low cancer rate in Iraq is unlikely because *H. pylori* is acquired late in life.

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A New *Helicobacter pylori* Vacuolating Cytotoxin Determinant, the Intermediate Region, Is Associated With Gastric Cancer

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Background & Aims: ***Helicobacter pylori*** **is the main cause of peptic ulceration and gastric adenocarcinoma. The vacuolating cytotoxin gene,** ***vacA*****, is a major determinant of virulence. Two naturally polymorphic sites in** ***vacA*****, the signal region and midregion, are well-characterized determinants of toxicity and markers of pathogenesis. The aim of this study was to characterize a new** ***vacA*** **polymorphic site, the intermediate (i) region. Methods: The** ***vacA*** **i-region was identified and characterized by constructing isogenic** ***vacA*** **exchange mutants and determining their vacuolating activity on HeLa, AGS, and RK13 cell lines. The** ***vacA*** **i-region types of** ***H pylori*** **isolates from patients undergoing routine endoscopy were determined by nucleotide sequencing and allele-specific polymerase chain reaction. Results: Two i-region types were identified, i1 and i2, and both were common among 42 Western clinical isolates. Interestingly, only naturally occurring s1/m2 strains varied in i-type; s1/m1 and s2/m2 strains were exclusively i1 and i2, respectively. Vacuolation assays showed that i-type determined vacuolating activity among these s1/m2 strains, and exchange mutagenesis confirmed that the i-region itself was directly responsible. Using a simple i-region polymerase chain reaction–based typing system, it was shown for 73 Iranian patients that i1-type strains were strongly associated with gastric adenocarcinoma ($P < 10^{-3}$). Finally, logistic regression analysis showed this association to be independent of, and larger than, associations of** ***vacA*** **s- or m-type or** ***cag*** **status with gastric adenocarcinoma. Conclusions: Together these data show that the** ***vacA*** **i-region is an important determinant of** ***H pylori*** **toxicity and the best independent marker of VacA-associated pathogenicity.**

BASIC–ALIMENTARY TRACT

It is estimated that more than half of the world's population is chronically infected with the gastric pathogen *Helicobacter pylori*. While infection with this gram-negative bacterium is the main cause of peptic ulcer disease and a significant risk factor for gastric adenocarcinoma (GC) and mucosa-associated lymphoid tissue lymphoma,[1–3] usually colonization is asymptomatic. *H pylori* strain virulence, host response, and environmental factors all contribute to infection outcome. A major *H pylori* virulence determinant is the vacuolating cytotoxin, VacA,[4,5] which induces cytoplasmic vacuolation in epithelial cells,[6] induces cytochrome *c* release from mitochondria leading to apoptosis,[7] causes gastroduodenal damage in a mouse model,[8] and increases gastric ulcer risk in *H pylori*–infected Mongolian gerbils.[9] The vacuolating activity of VacA has been extensively studied and shown to be dependent on the formation of oligomeric VacA structures in the host cell membrane that exhibit anion-selective channel activity.[10,11] The mechanism of subsequent vacuole formation and the cellular origin of the vacuoles from late endosomes has been characterized.[12–18]

vacA encodes a preprotoxin of ~139 kilodaltons,[19–22] which includes an amino-terminal signal peptide and a ~50-kilodalton carboxy-terminal domain that are both cleaved upon secretion to yield a mature toxin monomer of 87–95 kilodaltons.[21,23] Further processing may also occur at an exposed protease-sensitive loop into ~37-kilodalton N-terminal and ~58-kilodalton C-terminal fragments (p37 and p58, respectively).[21,24] Sequence polymorphisms occur throughout *vacA*, the 2 most diverse regions being the signal region, encoding part of the signal peptide and the N-terminus of the mature protein (which may be type s1 or s2), and the midregion, encoding part of the p58 domain (type m1 or m2).[25] Natural mosaicism occurs such that all combinations of signal region and midregion type exist.[25,26] Simple polymerase chain reaction (PCR)-based methodology[25] has led to many studies correlating *vacA* allelic type and upper gastrointestinal disease; and in many populations where *vacA* polymorphism has been found, *vacA* type s1/m1 strains have been shown to be associated with duodenal

Abbreviations used in this paper: GC, gastric adenocarcinoma; i, intermediate; nt, nucleotide; NUD, nonulcer dyspepsia; PCR, polymerase chain reaction.

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Table 1. *H pylori* Strains Used in This Study

Strain	Relevant characteristics[a]	Source
60190	Wild-type (ATCC 49503); *vacA* s1/i1/m1	Leunk et al[6]
60190/CAT	As 60190 except *cat* inserted immediately 3′ to *cysS*; Cm^R	Letley et al[38]
60190/A2	As 60190/CAT except *vacA* cluster A (nt 905–943) from Tx30a	This study
60190/B2	As 60190/CAT except *vacA* cluster B (nt 1039–1066) from Tx30a	This study
60190/C2	As 60190/CAT except *vacA* cluster C (nt 1112–1149) from Tx30a	This study
Tx30a	Wild-type (ATCC 51932); *vacA* s2/i2/m2	Leunk et al[6]
Tx30a/KAN	As Tx30a except *aphA* inserted into *fecE*; Km^R	Letley et al[38]
Tx30a/P1S1M1-A	As Tx30a/KAN except *vacA* replaced with s1/m1 allele from 60190 with the exception of nt 639–828; *cat* inserted immediately 3′ to *cysS*; Cm^R	Letley et al[38]
Tx30a/P1S1M1-B	As Tx30a/KAN except *vacA* replaced with s1/m1 allele from 60190 with the exception of nt 635–693 and 804–1149; *cat* inserted immediately 3′ to *cysS*; Cm^R	This study
93-67	Wild-type; *vacA* s1/i1/m2	This study
93-67/CAT	As 93-67 except *cat* inserted immediately 3′ to *cysS*; Cm^R	This study
93-67/i2-i1	As 93-67/CAT except *vacA* cluster B (nt 1039–1066) from 93-72	This study
93-67/i1-i2	As 93-67/CAT except *vacA* cluster C (nt 1112–1149) from 93-72	This study
93-72	Wild-type; *vacA* s1/i2/m2	This study
J226	Wild-type; *vacA* s1/i2/m2	This study
J226/KAN	As J226 except *aphA* inserted immediately 3′ to *cysS*; Km^R	This study
J226/i1-i2	As J226/KAN except *vacA* cluster B (nt 1039–1066) from 93-67	This study

[a]*vacA* nt positions are given relative to the equivalent position in Tx30a *vacA* (GenBank U29401).

and gastric ulceration[25,27–30] and GC,[30–34] making this allelic form an important marker of disease-associated *H pylori* strains. Functionally, s1-type strains are associated with vacuolating activity in vitro,[25,27] with the hydrophobic N-terminus of the toxin playing a vital role in this process.[35,36] In contrast, s2-type VacA is invariably non-vacuolating due to the presence of a hydrophilic N-terminal extension.[37–39] Midregion polymorphism determines the cell specificity of vacuolation by affecting toxin binding to epithelial cells, such that m1 forms vacuolate a wider range of epithelial cell lines than m2.[38,40–42]

In this study we describe a novel determinant of *vacA* toxicity, confirmed using an isogenic mutant approach, which we have called the intermediate (i) region. We show that 2 allelic variants of this region exist and are commonly found in strains from the United Kingdom, United States, and Iran. Furthermore, we describe a simple PCR-based typing system to determine i-region type and use this to show that the i-region type of an infecting strain is associated with the presence of GC. Finally, we show that *vacA* i-region typing is a better predictor of *H pylori* strain carcinogenic potential than current signal region and midregion typing systems.

Materials and Methods

Bacterial Strains, Plasmids, and Growth Conditions

H pylori strains and plasmid constructs used for mutation studies are shown in Table 1 and Supplementary Table 1, respectively (see supplemental material online at www.gastrojournal.org). Clinical *H pylori* isolates were cultured from antrum and body gastric biopsy specimens obtained with informed consent from patients undergoing endoscopy or, in the case of patients with adenocarcinoma, from resected stomach following gastrectomy. For the case-control study, *H pylori* was cultured from 32 patients with GC and 43 patients with nonulcer dyspepsia (NUD) in Tehran, Iran, from which genomic DNA was extracted and sent to the United Kingdom for PCR typing. *H pylori* was cultured on blood agar base 2 plates containing 7% (vol/vol) horse blood (Oxoid Ltd, Basingstoke, UK) for 48 hours at 37°C in a 5% CO_2 incubator. Broth cultures were grown in sulfite-free Brucella broth containing 0.2% (wt/vol) β-cyclodextrin (Sigma-Aldrich Co Ltd, Gillingham, Dorset, UK) microaerobically (Campypak Plus; BD Biosciences UK Ltd, Oxford, UK) at 37°C for 48 hours with shaking at 200 rpm. For broth culture supernatants, bacteria were removed by centrifugation (2890*g* for 10 minutes) followed by filtration (0.2 μm). *H pylori* water extracts were prepared as previously described.[38] Plasmids were propagated in the *Escherichia coli* K12 strain NovaBlue (Novagen; Merck Biosciences Ltd, Nottingham, UK) grown using Luria–Bertani media either in broth or on plates containing 1.5% (wt/vol) Agar No. 1 (Oxoid Ltd) supplemented with kanamycin, chloramphenicol (both 30 μg/mL), or carbenicillin (100 μg/mL) as appropriate.

Site-Directed Mutagenesis of vacA

As described in Results, a region of *vacA* sequence diversity was identified and shown to be a determinant of vacuolating activity. This region, called the i-region, encodes 3 clusters of polymorphic amino acid differences (clusters A, B, and C) between the deduced VacA sequence of toxigenic *H pylori* strain 60190 (s1/i1/m1) and non-toxigenic strain Tx30a (s2/i2/m2). To define which of these were important, i1-type clusters of 60190 *vacA* were individually replaced with the equivalent i2-type sequences from Tx30a *vacA*. To do this, plasmid pJR100,

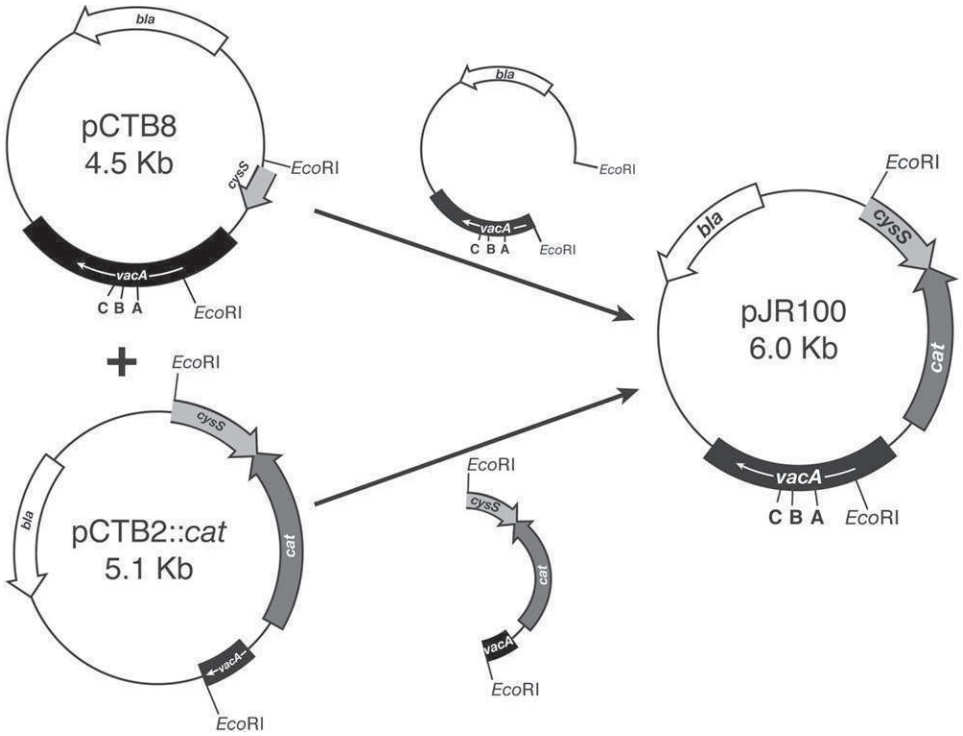

Figure 1. Construction of plasmid pJR100. Starting with pCTB8, containing 3′ *cysS* and 5′ *vacA* to nt 2032 from 60190,[19] the 0.7-kilobase *Eco*RI fragment containing 3′ *cysS* and the first 274 base pairs of *vacA* were replaced with the corresponding fragment from pCTB2::*cat*,[38] containing chloramphenicol acetyltransferase (*cat*; chloramphenicol resistance) inserted immediately downstream of *cysS*, to create pJR100. *bla*, β-lactamase (confers ampicillin resistance); *cysS*, cysteinyl/transfer RNA synthetase; *vacA*, vacuolating cytotoxin (*white arrow* denotes direction of transcription); A, B, and C represent the divergent clusters A, B, and C of the *vacA* i-region.

containing 3′ *cysS* (cysteinyl/transfer RNA synthetase) and 5′ *vacA* to nucleotide (nt) 2032 from 60190, with chloramphenicol acetyltransferase (*cat*) inserted immediately downstream of *cysS*, was constructed as shown in Figure 1. Site-directed mutagenesis was then performed on pJR100 as shown in Figure 2*A*, using the 5′ phosphorylated primers JR4F and JR4R (all mutagenesis primers are shown in Supplementary Table 2; see supplemental material online at www.gastrojournal.org), each containing half of the Tx30a i2-specific cluster A sequence (nt 913–943 in Tx30a) to be inserted at their 5′ end. Because cluster A from Tx30a introduced a new *Hin*dIII site, its presence was identified by PCR amplification of plasmid DNA using primers DL2 and VacR9, followed by restriction endonuclease analysis. The mutation was confirmed by nucleotide sequencing, and the resulting construct was called pJR101. Similar mutagenesis of pJR100 was performed using primers JR5F and JR5R for cluster B (nt 1039–1066) and JR6F and JR6R for cluster C (nt 1112–1149) to create pJR102 and pJR103, respectively. As before, mutations were identified by restriction analysis (cluster B removed a *Hin*dIII site present in 60190, and cluster C introduced a *Sau*3AI site) and confirmed by nucleotide sequencing. Finally, hybrid i-region alleles were introduced into 60190 chromosomal *vacA* by natural transformation, allelic exchange, and marker rescue (Figure 2*B*) and confirmed by restriction analysis of PCR products as before to create hybrid strains 60190/A2, 60190/B2, and 60190/C2.

To study the role of the *vacA* i-region among s1/m2-type strains, hybrids of the vacuolating s1/i1/m2-type strain 93-67 containing either i-region cluster B or C from the nonvacuolating s1/i2/m2-type strain 93-72 (93-67/i2-i1 and 93-67/i1-i2, respectively) were constructed. The first 1.3 kilobases of *vacA* from *H pylori* strain 93-67 were PCR amplified together with the upstream gene *cysS* using primers Cys1F and C0880, cloned into pGEM-T Easy (Promega [UK] Ltd, Southampton, UK), and a *cat* marker derived from plasmid pBSC103[43] was inserted immediately 3′ to *cysS* to create p93-67/CAT. i-region mutagenesis was performed by inverse PCR as before using primers JR5F and JR5R for cluster B and JR6F and JR6R for cluster C. Positive transformants were confirmed by nucleotide sequencing and named p93-67/i2-i1 and p93-67/i1-i2. These hybrid alleles were introduced into the chromosomal *vacA* gene of strain 93-67 as before to create strains 93-67/i1-i2 and 93-67/i2-i1. Control strain 93-67/CAT was derived by transforming 93-67 with p93-67/CAT. p93-72/KAN was constructed in a similar manner to p93-67/CAT except a kanamycin resistance marker (aminoglycoside phosphotransferase; *aphA*) derived from plasmid pILL600[44,45] was inserted 3′ to *cysS*. Primers JR7F and JR7R were used to introduce the i1-type cluster B into p93-72/KAN as before to create p93-72/i1-i2. After several unsuccessful attempts to transform strain 93-72 with p93-72/KAN and p93-72/i1-i2, these constructs were finally introduced into another nonvacuolating s1/i2/m2-type strain, J226, to create J226/KAN and J226/i1-i2.

Detection of VacA by Immunoblotting

VacA was detected in *H pylori* broth culture supernatant or water extract by immunoblotting as

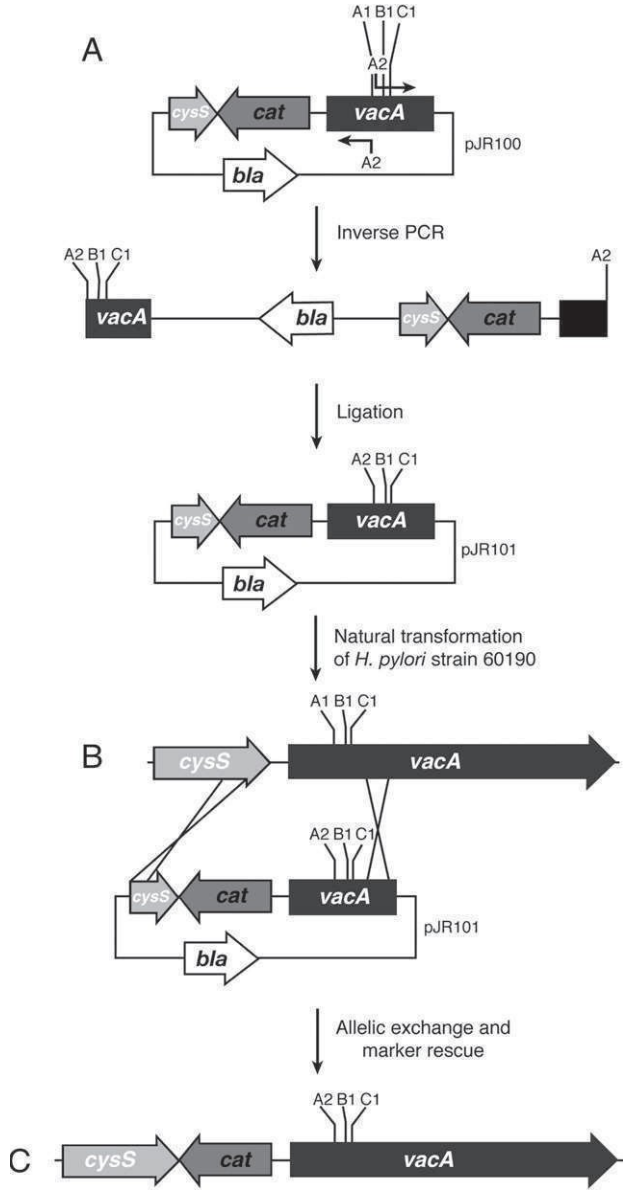

Figure 2. Construction of isogenic mutant strain 60190/A2 containing the Tx30a-specific cluster A sequence. (*A*) Site-directed mutagenesis was performed on pJR100 by inverse PCR using *Pfu* polymerase (Promega [UK] Ltd) and the 5′ phosphorylated primers, JR4F and JR4R, each containing half of the Tx30a i2-specific cluster A sequence to be inserted at their 5′ end (A2). The PCR product was recircularized by blunt-end ligation and transformed into competent *E coli* strain NovaBlue. The mutation was confirmed by nucleotide sequencing and the resulting construct called pJR101. (*B*) pJR101 was introduced into *H pylori* strain 60190 by natural transformation, allowing recombination to occur with the chromosomal *vacA* gene. (*C*) Following allelic exchange, hybrid strain 60190/A2 was isolated by selecting for chloramphenicol resistance. *bla*, β-lactamase (confers ampicillin resistance); *cat*, chloramphenicol acetyl transferase (confers chloramphenicol resistance); *cysS*, cysteinyl-tRNA synthetase; *vacA*, vacuolating cytotoxin; A1, B1, and C1, the i1-specific forms of divergent clusters A, B, and C of the *vacA* i-region.

previously described[38] using rabbit polyclonal antisera raised against recombinant p58 fragment from either the m1-type strain 60190 (Ab929)[46] or the m2-type strain Tx30a (Ab927) as appropriate, both kindly donated by Dr T. L. Cover (Nashville, TN).

Quantification of VacA

VacA protein was quantified in broth culture supernatants and water extracts by enzyme-linked immunosorbent assay as previously described.[38] Multiple samples were analyzed using anti-VacA antibodies Ab929 or Ab927, and results were expressed as mean A_{492} units.

Determination of Vacuolating Cytotoxin Activity

Vacuolation assays were performed on HeLa (ATCC CCL-2, a human cervical adenocarcinoma cell line), RK13 (ATCC CCL-37, a rabbit kidney cell line), and AGS (ATCC CRL-1739, a human GC cell line) epithelial cells as previously described.[38] Vacuolation was assessed visually by light microscopy.

vacA *i-Region Nucleotide Sequence Analysis*

The 0.5-kilobase region from 1131–1628 nt of *H pylori* 60190 *vacA* (GenBank accession no. U05676) was PCR amplified from genomic DNA prepared as described previously[19] using primers VacF1 and VacR9 (Supplementary Table 2; see supplemental material online at www.gastrojournal.org). PCR products were cloned into pGEM-T Easy and sequenced using M13F primer on an Applied Biosystems 3100 Genetic Analyzer (Biopolymer Synthesis and Analysis Unit, University of Nottingham). Nucleotide and deduced amino acid sequences were aligned using the Clustal V algorithm within MegAlign (DNAStar Inc, Madison, WI), including *vacA* from strains 60190 (s1/i1/m1) and Tx30a (s2/i2/m2; GenBank U29401) as controls.

Determining vacA *and* cagA *Genotype by PCR*

To allow rapid identification of i-region cluster C type, a simple PCR typing system was developed based on a conserved forward primer and specific reverse primers. Reactions contained 0.1–0.5 μg genomic DNA; 0.5 μmol/L of forward primer VacF1; 0.5 μmol/L of cluster C–specific reverse primer (C1R for i1 or C2R for i2; Table 2); 1× PCR buffer (Roche Diagnostics Ltd, Burgess Hill, UK); 200 μmol/L each of deoxyadenosine triphosphate, deoxythymidine triphosphate, deoxycytidine triphosphate, and deoxyguanosine triphosphate (Promega [UK] Ltd); and 2.5 U *Taq* DNA polymerase in a

Table 2. *vacA* i-Region Typing Primers

Primer name	Sequence (5′ to 3′)	Location (nt)[a]
VacF1	GTTGGGATTGGGGGAATGCCG	1131–1151
C1R	TTAATTTAACGCTGTTTGAAG	1536–1556
C2R	GATCAACGCTCTGATTTGA	1120–1138[b]

[a]Annealing site of primer in 60190 *vacA* (GenBank U05676).
[b]Annealing site of primer in Tx30a *vacA* (GenBank U29401).

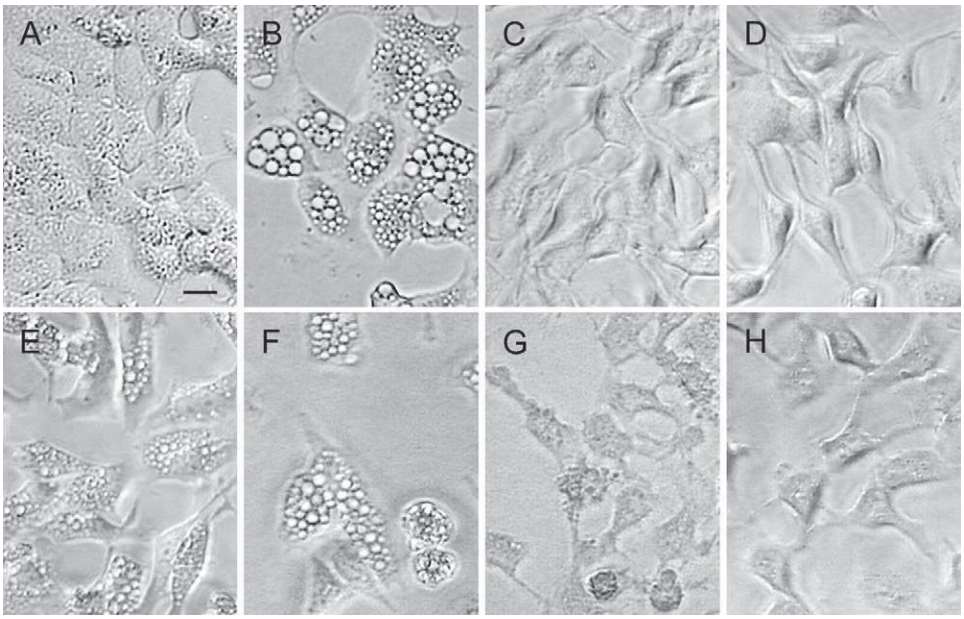

Figure 3. Vacuolating activity of control and isogenic *vacA* mutant strains. (*A* and *B*) RK13 cells or (*C–H*) HeLa cells grown in 96-well microtiter plates (10^4 cells/plate) were incubated overnight with *H pylori* water extracts of the following control and *vacA* mutant strains: (*A* and *C*) Tx30a/KAN, (*B* and *D*) Tx30a/P1S1M1-B, (*E*) 60190/CAT, (*F*) 60190/A2, (*G*) 60190/B2, and (*H*) 60190/C2. *Scale bar* = 15 μm.

25-μL volume. PCR was performed on a PCR Express thermocycler (Hybaid; Thermo Fisher Scientific, Basingstoke, UK) using 1 cycle of 95°C for 90 seconds; 35 cycles of 95°C for 30 seconds, 53°C for 60 seconds, and 72°C for 30 seconds; and 1 cycle of 72°C for 5 minutes. PCR products were separated by electrophoresis on 2% (wt/vol) agarose gels containing 0.5 μg/mL ethidium bromide in TAE buffer and examined under UV illumination. *vacA* signal region and midregion types and *cagA* status were also determined by PCR as described previously.[25,27,47]

Results

The Polymorphic vacA *Signal Region and Midregion Are Not the Only Natural Determinants of Vacuolating Activity*

Previously we have shown that natural polymorphism within the *vacA* signal region and midregion determines vacuolating activity and cell specificity, respectively, and replacing the s2/m2 *vacA* allele in the nontoxigenic strain Tx30a with s1/m1-type *vacA* from 60190 (hybrid strain Tx30a/P1S1M1) confers full vacuolating activity, indicating that toxin production and activity are not dependent on chromosomal elements outside of *vacA*.[37,38] When constructing this hybrid, a second independently derived transformant, Tx30a/P1S1M1-B, was also selected (the original reported hybrid strain is referred to here as Tx30a/P1S1M1-A). As previously reported, duplicate water extracts of Tx30a/P1S1M1-A induced vacuolation in HeLa, AGS, and RK13 cell lines similar to the 60190 *vacA* donor strain. In contrast, duplicate Tx30a/P1S1M1-B water extracts displayed a cell line–specific vacuolating activity, causing extensive vacuolation of RK13 cells (Figure 3*B*) but not HeLa cells (Figure 3*D*) or AGS cells (data not shown), despite VacA levels being higher in the Tx30a/P1S1M1-B water extracts as determined by VacA enzyme-linked immunosorbent assay (mean VacA enzyme-linked immunosorbent assay value [±SE], 0.464 ± 0.015 A_{492} units; mean Tx30a/P1S1M1-A value [±SE], 0.277 ± 0.018 A_{492} units) and immunoblotting (Figure 4). To confirm these findings, further duplicate water extracts were prepared for the hybrid strains. Again, Tx30a/P1S1M1-B water extracts only induced vacuolation in the RK13 cell line despite higher toxin levels relative to Tx30a/P1S1M1-A (means, 0.667 ± 0.001 and 0.332 ± 0.044 A_{492} units, respectively).

Sequence analysis of both hybrid strains showed that the majority of Tx30a *vacA* had been replaced with 60190 *vacA* (including the signal region and midregion). How-

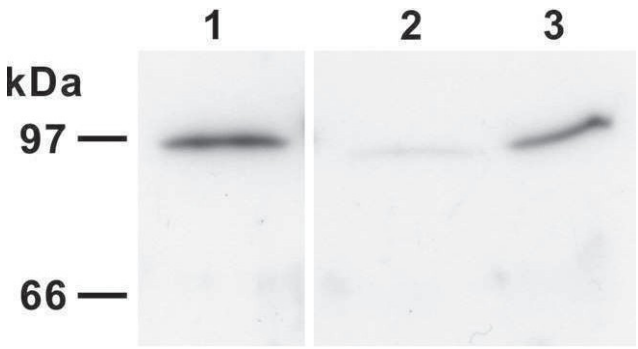

Figure 4. VacA immunoblot of Tx30a/P1S1M1 hybrid water extracts. Representative water extracts of *vacA* type s1/m1 control strain 60190 (*lane 1*) and the hybrid strains Tx30/P1S1M1-A (*lane 2*) and Tx30/P1S1M1-B (*lane 3*) were separated by sodium dodecyl sulfate/polyacrylamide gel electrophoresis on a single gel and immunoblotted with rabbit anti-VacA antiserum. VacA was detected as an immunoreactive band of ~95 kilodaltons.

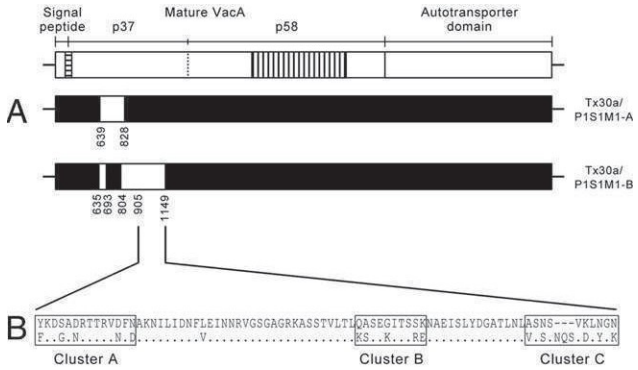

Figure 5. Determination of the *vacA* i-region. (*A*) Schematic representation of the *vacA* nt sequences of hybrids Tx30a/P1S1M1-A and Tx30a/P1S1M1-B. Sequence identity with the parental Tx30a *vacA* allele is shown as a *white bar*, and identity to the 60190 *vacA* allele is shown as a *black bar*. The first and last Tx30a-specific nucleotide positions are given relative to the Tx30a *vacA* sequence (GenBank U29401). The signal peptide, autotransporter domain, and mature toxin (including the p37 and p58 domains) are indicated. The signal region and midregion are shown by *horizontal* and *vertical bars*, respectively. (*B*) Deduced amino acid sequence alignment of the i-region defined by the first and last nonsynonymous nucleotide difference between Tx30/P1S1M1-A (top sequence; identical to 60190 *vacA*, GenBank U05676) and Tx30/P1S1M-B (bottom sequence; identical to Tx30a *vacA*). With one exception, amino acid substitutions fall into one of 3 clusters: A, B, or C.

ever, they differed in the extent of Tx30a sequence remaining, as shown in Figure 5*A*. In both hybrids, Tx30a-derived sequences were within the region encoding the p37 domain. Thus, *vacA* sequences other than the signal region and midregion determine full vacuolating activity.

Identification of a Novel Determinant of VacA Toxicity, the i-Region

Because region 635–693 nt of Tx30a sequence was present in both Tx30a/P1S1M1 hybrids (except for a nucleotide substitution at position 635 resulting in a conservative lysine to arginine substitution), we reasoned that this was not responsible for the difference in vacuolating activity observed. Similarly, we excluded nt 804–828, also present in both hybrid sequences. Thus, we hypothesized that codon changes within region 828–1149 nt were responsible for the difference in vacuolating activity observed between the 2 hybrids. This region was further delimited to a 245–base pair region from nt 905–1149, which encodes the nonsynonymous codon differences between the 2 hybrids, clustered within 3 subregions that we denote as clusters A, B, and C (Figure 5*B*).

To investigate whether differences in region 905–1149 nt affected vacuolating activity and to define which codon changes were important, clusters A, B, and C of the *vacA* allele of toxigenic strain 60190 were individually replaced with the type 2 variants of Tx30a by allelic exchange (see Materials and Methods) to create hybrids 60190/A2, 60190/B2, and 60190/C2. We have previously shown that inserting a chloramphenicol resistance marker upstream of the *vacA* promoter in allelic exchange experiments does not affect vacuolating activity,[38] and the same 60190/CAT control strain was used in these experiments. Vacuolating activity of hybrid and control strains was determined using triplicate water extracts and 48-hour broth culture supernatants on both RK13 and HeLa cell lines. While the 60190/CAT control induced vacuolation of both RK13 (data not shown) and HeLa cells (Figure 3*E*), 60190/B2 and 60190/C2 showed the same cell line specificity as Tx30a/P1S1M1-B, vacuolating RK13 cells (data not shown) but not HeLa cells (Figure 3*G* and *H*). In contrast, water extracts of 60190/A2 (n = 8) vacuolated both RK13 (data not shown) and HeLa cells (Figure 3*F*). Differences in vacuolating activity were not due to varying VacA levels, as determined by immunoblotting with anti-VacA antiserum Ab 929 (data not shown). Because replacing clusters B and C, but not A, affected vacuolating activity, we defined the *vacA* region from 1039–1149 nt in Tx30a as a novel determinant of vacuolating activity, which we called the i-region. The i-region sequence of toxigenic strain 60190 was defined as i1 type (nt 1463–1564 in this strain; GenBank accession no. U05676) and that of nontoxigenic strain Tx30a as i2 type. This region encoded residues 190–223 of the mature 60190 VacA peptide.

Both vacA *i-Region Types Are Common Among Western* H pylori *Clinical Isolates*

To investigate whether both *vacA* i-region types were common among clinical *H pylori* isolates, we sequenced this region from 42 strains (27 from Nashville, Tennessee, and 15 from Nottingham, England). Both i1- and i2-type alleles were present. Six strains had a variant i2-type allele in which the first 2 conservative substitutions of cluster B were i1-like (QASEKITSRE rather than KSSEKITSRE). Because this variant allele possessed the nonconservative i2-like substitutions in the rest of cluster B, and cluster C was also i2-like, these alleles were classified as being i2 type. The *vacA* signal region and midregion type of these strains was also determined by PCR as described previously.[25,47] Fourteen of 42 strains typed as s1/m1, and all were i-region type i1. Five of 42 strains typed as s2/m2, and all were i2 type. Twenty-two of 42 strains typed as s1/m2 and, unlike s1/m1 and s2/m2, had variable i-region types; 8 of 22 strains were i1 type, 12 of 22 were i2 type, and 2 of 22 were a recombinant type, which was i1 for cluster B and i2 for cluster C (i1-i2 type). Interestingly, one strain typed as the rare s2/m1 allele, and this was i2 type.

vacA *i-Region Type Correlates With Vacuolating Activity Independent of Midregion Type Among Clinical* H pylori *Isolates*

Next, we determined the vacuolating activity of the clinical *H pylori* isolates typed previously by incubating RK13 cells with duplicate water extracts from these

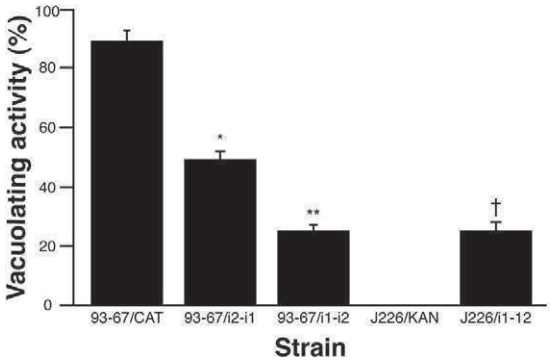

Figure 6. *vacA* i-region determines vacuolating activity. RK13 cells grown in 96-well microtiter plates (10^4/plate) were incubated overnight in duplicate with water extracts of s1/m2-type control and i-region isogenic mutant strains. The percentage of vacuolated cells was calculated by counting a minimum of 100 cells from random microscope fields. $^*P < 10^{-3}$, $^{**}P < 10^{-4}$ both compared with 93-67/CAT; $^{\dagger}P < 10^{-3}$ compared with J226/KAN (*t* test). Values are expressed as mean % ± SD.

strains and controls 60190 (Tox+) and Tx30a (Tox−). All *vacA* type s1/i1/m1 but none of the s2/i2 strains vacuolated RK13 cells. Of the 22 s1/m2 strains, all 8 i1-type strains but none of the 12 i2-type strains induced vacuolation ($P < 10^{-5}$, Fisher exact test). Interestingly, both strains with mixed i-type (i1-i2) induced a low level of vacuolation.

vacA *i-Region Type Determines Vacuolating Activity in s1/m2 Strains*

The effect of *vacA* i-type on vacuolating activity among s1/m2 strains was studied using a directed approach, by constructing isogenic *vacA* hybrids of vacuolating s1/i1/m2 strain 93-67 and nonvacuolating s1/i2/m2 strain J226 in which either i-region cluster B or C was exchanged (see Materials and Methods). Resulting hybrid strains were called 93-67/i2-i1, 93-67/i1-i2, and J226/i1-i2. Control strains 93-67/CAT and J226/KAN, containing chloramphenicol and kanamycin resistance markers, respectively, inserted upstream of the *vacA* promoter, were also constructed. Despite several attempts, we were unable to construct hybrid strain J226/i2-i1. We also tried to exchange the complete i-region between 93-67 and J226 but were unsuccessful. Next, we quantified hybrid and control strain vacuolating activity by counting vacuolated RK13 cells within random microscopic fields (minimum total of 100 cells) from duplicate experiments. 93-67/i2-i1 and 93-67/i1-i2 water extracts induced significantly less vacuolation of RK13 cells than control 93-67/CAT (Figure 6). While J226/KAN did not induce vacuolation of RK13 cells, J226/i1-i2 water extracts showed partially restored cytotoxin activity (Figure 6), inducing an equivalent level of vacuolation to 93-67/i1-i2 and naturally occurring type i1-i2 clinical isolates. Immunoblotting with Ab929 confirmed that VacA amounts in the water extracts of hybrid and control strains were equivalent (data not shown).

vacA *i-Type Can Be Accurately Determined by a Simple PCR-Based Typing System*

To allow rapid identification of i-region type among clinical isolates, we developed a simple PCR-based typing system. Because our isogenic mutant studies showed that i-region cluster C had a greater effect on vacuolating activity, and sequence data showed that most strains type the same for clusters B and C, we designed our typing system to differentiate between cluster C types. For this we used the forward primer VacF1, annealing to a conserved sequence upstream of the i-region, and designed reverse primers specific for either the i1- or i2-type cluster C sequence (C1R and C2R, respectively; see Table 2). PCRs were performed using genomic DNA from the 42 clinical isolates for which we had previously determined i-region type by nucleotide sequencing. We performed 2 PCRs for each sample: an i1-specific reaction using VacF1 and C1R and an i2-specific reaction using VacF1 and C2R (predicted product sizes of 426 and 432 nt, respectively). Using this typing system, we correctly identified i-region type for all 42 Western clinical strains tested, as confirmed by our previous nt sequence data.

Infection With a vacA *i1-Type Strain Is Associated With GC*

Studies have shown that infection with *vacA* s1/m1-type strains is associated with GC.[30–34] We aimed to determine whether *vacA* i-region type was also associated with gastric cancer. In a case-control study, genomic DNA was prepared from clinical *H pylori* isolates from Iran, including 32 patients with GC and a control group of 43 patients with NUD, and *vacA* signal, i-region and midregion types, and *cagA* status were determined by PCR as described in this study and previously.[25,27,47] Two strains had unusual PCR product sizes for either the *vacA* signal region or midregion (1 GC and 1 NUD) and were excluded from further analysis. The results for the remaining 73 strains are shown in Table 3. The Iranian strains showed a similar distribution of *vacA* allelic types as the Western strains studied earlier, in particular s1/m1

Table 3. *vacA* Allelic Types and *cagA* Status of *H pylori* Strains Isolated From Iranian Patients

	vacA i-region type (no. of patients)			
Gene	i1	i2	i1 and i2	Total
vacA				
s1/m1	21	1	9	31
s1/m2	9	14	3	26
s1/(m1 and m2)	0	0	2	2
s2/m2	0	13	1	14
Total	30	28	15	73
cagA				
Positive	29	19	15	63
Negative	1	9	0	10
Total	30	28	15	73

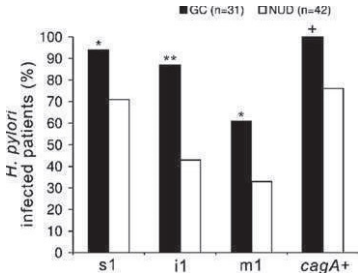

Figure 7. Association of *vacA* allelic type and *cagA* status of the infecting *H pylori* strain and GC in Iranian patients. The percentage of *H pylori*–infected patients possessing a *vacA* s1, i1, or m1 or a *cagA*-positive strain is shown for both GC (*black bars*) and NUD (*white bars*) groups. $^{*}P < .05$, $^{**}P < .0005$, $^{+}P < .005$ (Fisher exact test).

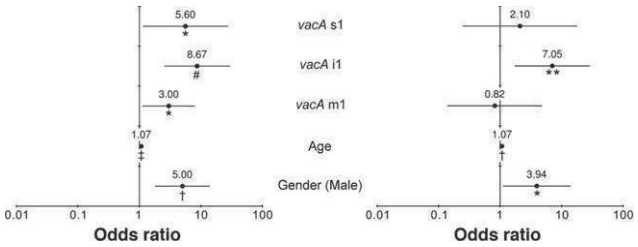

Figure 9. Logistic regression analysis of the effect of *H pylori vacA* allelic type, patient age, and sex on the occurrence of GC. Univariate (*left*) and multivariate (*right*) logistic regression analysis was performed on the Iranian strain typing data and the calculated odds ratios plotted along the horizontal axis for each variable (vertical axis). *Error bars* represent 95% confidence intervals. The final multivariate logistic regression model was obtained using a stepwise elimination method, removing *vacA* m-type first and then *vacA* s-type. $^{*}P < .05$, $^{**}P < .01$, $^{\dagger}P < .002$, $^{\#}P < .001$, $^{\ddagger}P < .0005$.

strains were predominantly i1 type, s2/m2 strains were all i2 type, and the s1/m2 group varied in i-region type. Fifteen strains gave a PCR product of the expected size for both i1 and i2 specific reactions (11 GC and 4 NUD). To check whether this was due to poor primer specificity resulting in annealing to the same *vacA* allele, both i1 and i2 PCR products were sequenced for 7 of these patients. All 14 i-region PCR product sequences were unique, and i1 and i2 sequences from the same patient were more divergent (mean pairwise identity ± SD for i1 vs i2 = 90.4% ± 1.7%) than sequences of the same i-type from different patients (mean pairwise identity for i1, 95.8% ± 1.5%; for i2, 94.8% ± 1.7%), confirming that both i-region types were present. Because genomic DNA had been prepared from colony sweeps from biopsy cultures, the most likely explanation for mixed i-region type is multiple infection within the respective patients. Indeed, 2 strains also gave mixed midregion typing results.

As observed for other populations, GC in this Iranian patient group was significantly associated with the presence of the *vacA* s1 allele ($P < .05$, Fisher exact test), the m1 allele ($P < .05$), and *cagA* ($P < .005$) in the infecting *H pylori* strain (Figure 7). Interestingly, the presence of a *vacA* i1-type strain was also significantly associated with GC ($P < .0005$; Figure 7), and this association remained significant when patients with multiple i-type strains were excluded: 16 (80%) of 20 patients with GC compared with 14 (37%) of 38 patients with NUD ($P < .005$). Subgroup analysis showed that among patients infected with a *vacA* s1-type strain, the i1 allele was still significantly associated with GC ($P < .02$) in contrast to the *vacA* m1 allele and *cagA* status (Figure 8*A*). Similarly, only i-region type was significantly associated with GC independent of m-type ($P < .01$, Figure 8*B*) and *cagA* status ($P < .01$; Figure 8*C*), and none of the allelic markers were predictive of disease independent of i-region type (Figure 8*D*).

Finally, to further assess the relative importance of *vacA* signal, i-region, and midregion polymorphisms as GC risk factors, we used logistic regression analysis, including patient age and sex in our model (*cagA* status was not included because all patients with GC were infected with *cagA*-positive strains). Inclusion of age and sex was particularly important because our groups were not matched for these factors (the 32 patients with GC had a median age of 60 years with 65.6% male, and the 43 patients with NUD had a median age of 36 years with 30.2% male). Univariate analysis showed that infection with a *vacA* s1, m1, or i1-type strain, age, and sex (male) were all significantly associated with GC (Figure 9, *left panel*). However, multivariate analysis revealed that only infection with a *vacA* i1-type strain, age, and sex were independently associated with an increased odds of developing GC (Figure 9, *right panel*), suggesting

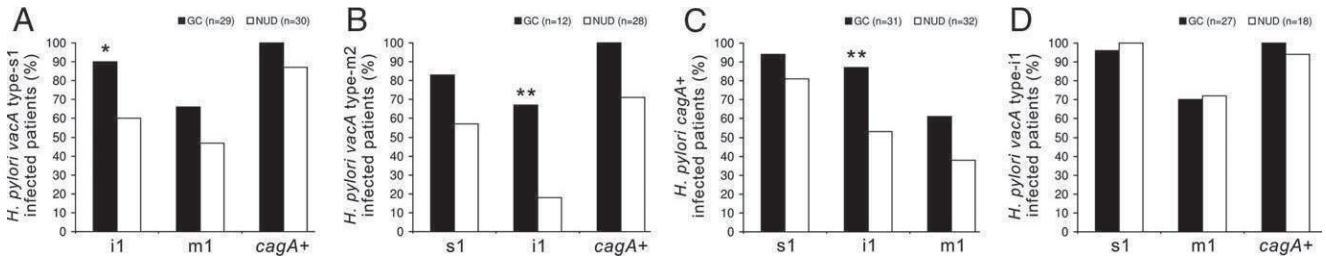

Figure 8. Subgroup analysis of the association between *vacA* allelic type and *cagA* status of the infecting *H pylori* strain and GC in Iranian patients. Patient data were divided into the following subgroups based on the allelic type of the infecting *H pylori* strain: (*A*) *vacA* s1-type only, (*B*) *vacA* m2-type only, (*C*) *cagA*-positive only, and (*D*) *vacA* i1-type only. For each subgroup, the percentage of patients with GC (*black bars*) and NUD (*white bars*) infected with a strain also possessing the remaining allelic types described is shown. $^{*}P < .02$, $^{**}P < .01$ (Fisher exact test).

that i-region type is the best *vacA* determinant for assessing GC risk. Finally, a rare finding from this population was the presence of a natural s1/m1 strain that was i2 type, and interestingly this was identified in a patient from the non-cancer (NUD) group.

Discussion

The vacuolating cytotoxin, VacA, is a major virulence determinant of *H pylori*. Although all strains possess *vacA*, the gene encoding this toxin, and most secrete VacA protein, not all strains induce cytoplasmic vacuolation of HeLa cells in vitro, and those that do are more likely to be isolated from patients with disease.[4–6,48] Previously, we showed that *vacA* is polymorphic and described a mosaic structure based on 2 regions of greatest diversity: the signal (s) region and the mid (m) region.[25] We and others have shown that the signal region affects the vacuolating activity of the toxin: a 12–amino acid hydrophilic extension on the s2 form blocks activity,[37–39] although not all s1 forms are cytotoxic. The midregion determines the cell specificity of the toxin,[38,40,41] affecting binding to host cells.[40] In this study, we identified a third polymorphic determinant of vacuolating activity, located between the signal region and midregion within the p37 domain, which we termed the i-region. We described 2 i-region types, i1 and i2, both common among clinical *H pylori* isolates. Among Western strains studied, s1/m1-type *vacA* alleles were invariably i1 type and showed vacuolating activity, while s2/m2-type alleles were i2 type and were nonvacuolating. Importantly, s1/m2-type alleles varied in their i-region status, and this affected their ability to induce vacuolation of RK13 cells in vitro; s1/i1/m2 strains induced vacuolation, whereas s1/i2/m2 strains did not. This may have important implications for previous studies relating *vacA* midregion type to disease because s1/m2 strains may have differed in i-region type and vacuolating activity. While the effect of i-type on the vacuolating activity of s1/m2 VacA was studied using the rabbit kidney cell line RK13, it is important to note that the s1/m2 strain 95-54, first shown to be active on RK13 but not HeLa cells by Pagliaccia et al,[40] was also shown to induce vacuolation of primary human gastric cells. 95-54 *vacA* has a mosaic i-region, encoding an i2-like cluster B and an i1-like cluster C. Thus, its vacuolating activity on RK13 cells is consistent with our data. Furthermore, purified 95-54 VacA caused less vacuolation of RK13 cells than s1/i1/m1-type VacA from strain CCUG17874,[40] and it cannot be ruled out that i-region differences were a contributing factor.

We confirmed that i-type determined vacuolating activity using an isogenic mutant approach, which also allowed us to examine polymorphic differences within the i-region in greater detail. Amino acid substitutions occur within 3 clusters, each containing 5 substitutional differences. All 5 differences in cluster A are conservative based on PAM250 substitutional matrix scores,[49] and site-directed mutagenesis showed these do not affect vacuolating activity. Clusters B and C both contain 3 nonconservative substitutions, and we speculate that it is these differences that affect the vacuolating activity of the toxin. Cluster C also contains a 3-residue insertion/deletion, which may account for its greater effect on vacuolating activity than cluster B. It is clear that polymorphic differences in the i-region affect the vacuolating activity of VacA, and we next plan to determine why this is so. Several clues from the published literature are discussed in the following text.

The N-terminal 422 residues of VacA, consisting of p37 and the first 111 residues of p58,[50] represent the minimal domain required for vacuolation when expressed intracellularly. This includes the i-region but not the midregion. In-frame deletions within the p37 domain, including residues 112–196 of mature 60190 VacA, which overlap with cluster B (190–199), abolish vacuolating activity by disrupting interaction between p37 and p58, preventing assembly of oligomeric VacA structures.[35,51] The exact location of residues required for p37 interaction with p58 is still unclear, because deletion of residues 112–196 will likely influence the conformation of other p37 regions, including the overlapping i-region.

When VacA interacts with lipid membranes, several regions are protected from protease digestion, suggesting they are either transmembranous or tightly bound to the membrane surface.[52] One such "protected" region is located just 6 residues downstream of i-region cluster B and includes cluster C. Such a location may allow polymorphic differences in the i-region to affect VacA oligomerization in a similar manner to that shown for another "protected" region, residues 49–57.[53] Alternatively, a transmembrane location may mean that i-region differences affect VacA channel activity or selectivity. The closer membrane association of cluster C than B could explain why cluster C substitution had a greater effect on vacuolating activity.

Polymorphic differences within the p58-located midregion have been shown to influence the cell specificity of VacA,[38,40–42] which correlates with cell surface binding.[40] However, these studies do not exclude the presence of other VacA regions, which may contribute to the specificity of the toxin. Indeed, none of these studies examined midregion differences in the context of an i2-type strain. Both recombinant p37 and p58 domains interact with lipid bilayers[54] and bind to HeLa cells,[46,55] and their interaction is required for increased binding, internalization, and vacuolating activity.[55] Thus, polymorphic differences in p37, such as those in the i-region, could influence toxin binding and internalization rather than channel activity.

A major finding of this study was the significant association of i1-type strains with GC in *H pylori*–infected patients from Iran. Although such an association requires confirmation by further studies using other pop-

ulations where *vacA* diversity exists, this result is potentially clinically important. Previous studies have shown an association between *vacA* s1/m1 strains and GC.[30–34] However, in all these studies, some s1/m2 strains were also isolated from patients with cancer. Here we have found that strains typing as i1 include all pathogenic s1/m1 strains and those s1/m2 strains that display vacuolating activity in vitro. Thus, determining i-region type may be sufficient for identifying all pathogenic forms of VacA. This conclusion is supported by logistic regression analysis of our *vacA* typing data, which indicates that, of the 3 *vacA* polymorphic sites, only i-region type is a significant, independent marker of GC-associated strains.

Supplementary Data

Supplementary data associated with this article can be found, in the online version, at 10.1053/j.gastro.2007.06.056.

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Original Article

A Study of *Helicobacter Pylori*-associated Gastritis Patterns in Iraq and Their Association with Strain Virulence

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ABSTRACT

Background/Aim: *Helicobacter pylori* (*H. pylori*) infection causes peptic ulceration and gastric adenocarcinoma. In Iraq, gastric cancer is rare. We investigated whether infected adults had the antral-predominant pattern of *H. pylori*-associated gastritis, which does not predispose to cancer. **Materials and Methods:** We evaluated histopathological changes by the Sydney scoring system in gastric biopsies taken from 30 *H. pylori*-infected adults and studied the correlation of these changes with the virulence factors. The Mann–Whitney test was used for the comparison of histopathological data. The presence or absence of each pathological index was evaluated with respect to the possession of virulence factors by the infecting *H. pylori* strain using the χ^2 test. **Results:** Gastric lymphocyte infiltration was more prominent in the antrum ($P = 0.01$). Neutrophil infiltration was mild and gastric mucosal atrophy was rare. No relationship was found between virulence factors and histopathological changes. **Conclusions:** The mild pathology and antral-predominant gastritis help explain the low cancer rate in Iraq.

Key Words: Antral-predominant gastritis, *H. pylori*, Iraq, low cancer rate

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Helicobacter pylori infection causes peptic ulceration and gastric adenocarcinoma. In Iraq, despite the early acquisition of *H. pylori* infection,[1] gastric cancer is unusually rare.[2] Despite the geographical proximity of Iraq, Turkey and Iran, the incidence of gastric cancer differs hugely among these countries, being ≤ 5/10^5, 8.9–14.1/10^5 and 38–69/10^5, respectively.[2] Virulence factors of Iraqi *H. pylori* strains appear more closely related to Western countries and unlikely to explain the low cancer rate completely.[3] Nothing is known about the distribution of *H. pylori*-associated inflammation in Iraqi patients or its severity. Thus, we now aim to assess the degree and distribution of inflammation in the stomachs of Iraqi people and the relationship between *H. pylori* virulence factors (*vacA*, *cagA* and *dupA*) and histopathological changes.

MATERIALS AND METHODS

The study protocol was approved by the ethical committee of the University teaching hospital where biopsies were collected. Samples for histopathology were obtained from 30 adult subjects with dyspepsia and without peptic ulceration. Two biopsies from the antrum and two from the corpus were taken and fixed in 10 mL buffered 10% formalin for histopathological examinations. The histological findings from the sections were scored according to the updated Sydney system of classification and grading of gastritis.[4] These slides were graded for the following features: *H. pylori* density, neutrophilic activity, lymphocytic infiltration and glandular atrophy. A visual analogue scale was used to assess the severity of the inflammatory changes and grading was performed as follows: l, mild; 2, moderate; 3, severe. All biopsies had Alcian blue (pH 2.5) to confirm the presence of metaplasia. Because our main aim was to determine the pattern of gastritis, we did not use any special stain to demonstrate *H. pylori* and we did not include *H. pylori* density in any further analysis.

Polymerase chain reaction-based genotyping of *H. pylori* isolates was performed on DNA extracted from bacteria or directly from biopsies.[3]

The Mann–Whitney test was used for the comparison of histopathological data. The presence or absence of each pathological index was evaluated with respect to the possession of virulence factors by the infecting *H. pylori*

Table 1: *H. pylori*-associated gastritis in biopsies taken from the gastric antrum and corpus

Variables	Antrum				Corpus				*P*-value
	Score 0	Score 1	Score 2	Score 3	Score 0	Score 1	Score 2	Score 3	
Lymphocyte infiltration (*n*)	0	10	14	5	0	20	7	2	0.016*
Neutrophil infiltration (*n*)	12	15	2	0	17	11	1	0	0.19
Intestinal metaplasia (*n*)	27	1	1	0	26	3	0	0	0.68
Atrophy (*n*)	28	1	0	0	28	1	0	0	1

*Significant *P*-value measured by the Mann–Whitney *U* test.

strain using the χ^2 test.

RESULTS

Pathological changes were observed in biopsies from 29/30 patients. The biopsies from 1/30 were histologically normal despite *H. pylori* being cultured. Among the 29 patients with inflammation in gastric biopsies, lymphocyte infiltration was more prominent in the antrum (Mann–Whitney *U* test, $P = 0.01$). There was no significant difference in neutrophil infiltration or mucosal atrophy between the antrum and the corpus. However, neutrophil infiltration was mild and was virtually absent (score 0) in 41% of the antral biopsies and 59% of the corpus biopsies. Furthermore, histological evidence of mucosal atrophy was seen in only 1/30 antral biopsies (patient age = 40 years) and 1/30 corpus biopsies (patient age = 64 years). In both the cases, it was mild. Intestinal metaplasia was found in 2/30 (6.8%) and 3/30 (10%) biopsies taken from the antrum and the body, respectively [Table 1].

The virulence factors of Iraqi *H. pylori* strains and their relationship with clinical outcome were studied previously.[3] In this study, we aimed to explore the relationship between individual virulence determinants and gastric histopathological changes in the gastric antrum and corpus. 66.6% (20/30) and 30% (9/30) of Iraqi strains typed positive for *cagA* and *dupA*, respectively. The distribution of *vacA* allelic types among *H. pylori* strains is shown in Table 2. For the *vacA* s, i and m regions individually, 28/30 (93.3%) isolates were the type s1, 8/30 (26.6%) isolates were the type i1 and 15/30 (23.3%) isolates were the type m1. To test whether the presence of specific genotype correlated with histopathological changes in the antrum and the corpus, our data were restratified according to the histological changes. No significant associations were found between *vacA*, *cagA* and *dupA* and histopathological scoring.

Table 2: Distribution of the *vacA* allelic types in Iraqi strains

	s1/i1/m1	s1/i1/m2	s1/i2/m1	s1/i2/m2	s2/i2/m2
n (%)	7/30 (23.3)	1/30 (3)	8/30 (26.6)	12/30 (40)	2/30 (6)

DISCUSSION

H. pylori is a risk factor for gastric cancer and gastric lymphoma.[5] Around the world, despite the high frequency of *H. pylori* infection, the incidence of gastric cancer is discordant.[5] The annual incidence rate of gastric cancer is very high in Japan and China, but *H. pylori* seropositivity is low.[5] In contrast, in India, seropositivity of *H. pylori* is very high but the annual incidence of gastric cancer is low.[5] One possible explanation is the difference in the gastritis pattern as pangastritis and corpus-predominant gastritis are associated with an increased risk of gastric mucosal atrophy and increased risk of cancer.[6,7] In two studies conducted in Japan and Iran, where there is a high gastric cancer rate, it was shown that *H. pylori* infection was strongly associated with chronic gastritis and that histological corpus gastritis was found with a high frequency.[8,9] On the other hand, in India and UAE, where the gastric cancer rate is low, the distribution pattern of gastritis was found to be antral-predominant.[10,11] Potentially, the low cancer rate in Iraq could be explained by antral-predominant gastritis being the common pattern and/or by inflammation being mild. In a study conducted in Iran, a neighbouring country to Iraq where the gastric cancer rate is very high,[2] it was found that mononuclear cell infiltration was similar throughout the stomach. On an average, patients had pangastritis.[9] In Iraq, we have shown that there is antral-predominant mononuclear cell infiltration. These findings are in agreement with the results from Kenya, an African country with a very low gastric cancer rate, where there is antral-predominant gastritis with significant discordance in the severity of graded variables between antral and corpus biopsies.[12] Furthermore, in Iran, histological evidence of mucosal atrophy was seen in 39% and 22% of the antral and corpus samples, respectively.[13] In another study conducted in Turkey, it was found that 43% of the *H. pylori*-infected subjects had atrophic gastritis.[14] In our study, glandular atrophy was found in only one (3%) specimen taken from the antrum and one from the corpus. Thus, despite the early acquisition of *H. pylori*,[1] the presence of atrophy appears rare in Iraq. We speculate that this antral-predominant gastritis and low glandular atrophy rate in Iraq might contribute to the low cancer rate.

Studies from North America revealed that infection with

cagA-positive *H. pylori* strains increases the risk of atrophic gastritis and gastric cancer.[15] However, this was not confirmed in several studies in Asian populations.[16] European studies showed a strong significance of the *vacA* m1 genotype with respect to epithelial damage, neutrophilic and lymphocytic infiltrates, atrophic gastritis and intestinal metaplasia. In addition, it was shown that severe damage to the gastric epithelium is associated with *vacA* s1/m1 mosaicism.[17] In India, it was shown that the s1a/m1 and s1a/m2 *H. pylori* vacA genotypes are significantly associated with severe chronic gastritis and gastric epithelial cell apoptosis than s2/m2.[18] In this paper, we aimed to study the correlation between individual virulence markers and histopathological changes in Iraq. In contrast to other studies,[17-19] no correlation between virulence factors and histopathological changes could be observed. In a study conducted in India, risk factors for gastric diseases were assessed in two populations with different incidences of gastric disease. It was shown that diet was the primary factor relating to the differences in the prevalence of duodenal ulcer. In Iraq, virulence factors of *H. pylori* were studied but these could not explain the low cancer rate. Probably studying other risk factors such as diet and smoking may help explain the mild pathology.

Our study has limitations, in particular that the sample size is small. However, because of the low rate of atrophy, it is unlikely to be misleading. Why gastric mucosal atrophy is uncommon in Iraq needs further study. However, our results raise the possibility that it may, in part, be due to the antral-predominant infiltration pattern seen in *H. pylori*-infected Iraqi population.

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Abstracts

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