Clinical Relevance Of

Helicobacter pylori Genotypes

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by

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STATEMENT OF PERSONAL INVOLVEMENT

The work described in this thesis was carried out during the tenure of a Research Fellowship in the University Department of Medicine at the Leicester Royal Infirmary Hospital, Leicester, and describes wholly original work. All the work described was performed by the author with the following exceptions :

1. The biopsies for histology samples were processed by the routine histopathology laboratory and all gastric histopathology assessed and interpreted by Dr. K.P.West.
2. Technical assistance and guidance was provided by J.Stephens, with the laboratory techniques including culture of *H. pylori.*
3. Some of the endoscopies and biopsy samples were carried out by Registrar colleagues and endoscopy nurses.

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Chapter 1

1.1 General Introduction.

Spiral organisms were first noted in the gastric mucosa of mammals at the end of the last century, but the significance of these organisms was not fully appreciated until the early 1980's. In 1983, Warren and colleagues isolated S-shaped bacteria from the human gastric mucosa, which were initially named *Campylobacter pyloridis*, (Marshall *et al*, 1984) and later renamed *Helicobacter pylori* [*H. pylori*] (Goodwin *et al*, 1989). The isolation of *H. pylori* was the beginning of a new understanding of the aetiology and pathogenesis of gastritis, peptic ulcer disease (PUD) and gastric cancer.

PUD is a common disorder of the gastrointestinal tract, with approximately 10% of the Western population being afflicted by the disease at some time in their life (as referenced by Berstad *et al*, 1993). *H. pylori* is now recognised as a major aetiological factor in the development of chronic gastritis and PUD; 90% of duodenal ulcers and approximately 70% of gastric ulcers are caused by *H. pylori* infection. Successful eradication of the bacterium results in healing of the ulceration and reduces the rate of relapse. The importance of *H. pylori* in the development of gastroduodenal pathology extends beyond PUD. *H. pylori* is classified as a Group I carcinogen (International Agency for Research on Cancer, 1994), and eradication of *H. pylori* in patients with low grade B cell lymphoma of the mucosal associated lymphoid tissue (MALT) leads to regression of the lymphoma (Wotherspoon *et al*, 1993).

Despite the well-established causal relationship between *H. pylori* and gastroduodenal pathology the pathogenic mechanisms of *H. pylori* induced disease are still poorly understood. Although the majority of patients with significant gastritis and PUD will be infected by *H. pylori*, only a minority of infected individuals will go onto develop more

serious gastrointestinal pathology. Interplay of host, bacterial and environmental factors are likely to be important.

Certain phenotypic characteristics differ between strains of *H. pylori*, and are thought to be potentially important in bacterial pathogenesis. Two proteins have been shown to be associated with the presence of PUD; the vacuolating cytotoxin (VacA) and the cytotoxin associated gene protein (CagA). The genes encoding VacA and CagA have been identified (Cover *et al*, 1994; Tummuru *et al*, 1993). Several groups have demonstrated that the vacuolating cytotoxin and CagA are potentially important factors in identifying virulent strains of *H. pylori* (Covacci *et al*, 1993; Tummuru *et al*, 1993; Atherton *et al*, 1995).

Identification of these 'virulence' factors is an important finding. However, further work is needed to look at these genes, the gene products and the effect on the host within different study populations. Clarification of their potential role in the pathogenesis of inflammation and gastroduodenal disease is essential if *vacA* and *cagA* are to have any clinical application.

1.2 Discovery.

Spiral organisms were first described in mammalian stomachs at the end of the last century when an Italian pathologist, Bizzozero, noted spirochetes in the gastric mucosa of dogs (as referenced by Buckley *et al*, 1998). Several references were subsequently made to gastric spiral shaped organisms, although the bacteria were often mis-identified. In 1906, Krienitz identified gastric spirals in patients with ulcerating gastric carcinomas (as referenced by Buckley *et al*, 1998). It was also noted that these organisms were rare in the gastric mucosa of normal individuals. In 1940, Freedburg and Barron reported that spirochetes were present in patients with benign and malignant gastric ulceration, and it was suggested that the organisms were a possible aetiological agent in peptic ulcer disease (Freedburg *et al*, 1940). A subsequent study in America failed to find the bacteria in over a thousand gastric biopsy samples (Palmer *et al*, 1954). Twenty years later, Steer and colleagues noted the presence of bacteria, using an electron microscope, on the surface of epithelial cells in 80% of patients with gastric ulcers; the bacteria were thought to be a *Pseudomonas* species (Steer *et al*, 1975).

In the early 1980's Warren and colleagues observed S-shaped organisms in gastric biopsy specimens and noted that the presence of bacteria were associated with a marked infiltration of granulocytes (Warren *et al*, 1983). Further work by Warren and Marshall in Australia led to the isolation of bacteria from gastric tissue specimens. Initial attempts to isolate the bacteria had failed and it was not until after a prolonged period of culture, where the plates were left in an incubator over a bank holiday weekend, that the bacteria was successfully cultured. Bacterial colonies were observed, and the organism described as *Campylobacter* - like, since they were curved, microaerophilic Gram-negative bacteria that resembled other *Campylobacter*. The bacterium was initially named *Campylobacter pyloridis* (Marshall *et al*, 1984) and then changed to *Campylobacter pylori* (Marshall *et al*, 1987). As differences between the new species and *Campylobacter* became more apparent,

the organism was subsequently classified within a new genus, *Helicobacter*, based on unique ultrastructure, fatty acid and genetic characteristics (Goodwin *et al*, 1989).

1.3 Bacteriology

1.3.1 Taxonomy.

Study of *Campylobacter pylori* and its ultrastructure lead to the finding that several features of the bacterium differed to the other members of the *Campylobacter* genus. Review of the taxonomic data lead to the formation of a new genus - the *Helicobacters* (Goodwin *et al*, 1989) - to mean a spiral rod. Several key features ascribed to the genus were; (i) multiple sheathed flagella, with terminal bulbs, as means of cell motility, (ii) the major isoprenoid quinone is menaquinone (MK-6) and lacks the methylated MK-6, (iii) an external glycocalyx produced *in vitro* in liquid media, (iv) specific growth requirements and enzyme capabilities, (v) a characteristic cellular fatty acid profile and (vi) Guanine and Cytosine content of the chromosomal DNA of 35-44mol %.

Since the formation of the *Helicobacter* genus, more than 18 species have been identified. The *Helicobacters* colonize the alimentary tract of diverse animal hosts and can be divided into gastric and non-gastric. *H. pylori* is the best known gastric pathogen, but others include *H. mustelae* (ferrets), *H. felis* (cats and dogs) and *H. acinonyx* (cheetahs). *H. hepaticus* is a non-gastric *Helicobacter* species, isolated from the intestine and liver of mice (for review see International Agency for Research on Cancer 1994)

1.3.2 The genome.

In comparison with other bacterial species, *H. pylori* displays an unusual degree of diversity at the genetic level (Jiang *et al*, 1996; Campbell *et al*, 1997; Hazell *et al*, 1997). However, the overall gene organisation and protein sequences are relatively conserved, with recent analysis of 2 unrelated isolates showing only 7% of proteins were strain specific (Alm *et al*, 1999). The genome DNA of *H. pylori* is a single circular molecule which is 1.4 - 1.73 Mb in size and has recently been sequenced (Tomb *et al*, 1997). The genome of H. *pylori* is thymine and adenine rich with a relatively narrow range of guanine

- cytosine content of on average 39%. Five regions within the genome are significantly different in their G+C content. Two of these regions contain genes involved in DNA processing and possibly in toxin transport, and a third region is termed the '*cag* pathogenicity island' (PAI). One thousand, five hundred and ninety predicted genes have been identified, with an average size of 945 bp. All *H. pylori* have now been cloned, some examples of which are: the urease genes, two flagellin genes, the cytotoxic *vacA* gene, a cytotoxin production associated gene and a heat shock protein encoding gene (for references see International Agency for Research on Cancer, 1994). The vast number of potential gene products is a likely contributor to the complexity of the host-pathogen interaction. Recombination of the large number of surface exposed proteins may be one mechanism contributing to antigenic variation and evasion of the host's defense mechanisms. *H. pylori*, *Escherichia coli* and *Haemophilis influenzae* are classified as Proteobacteria (a large division of Gram negative bacteria). Although many *H. pylori* proteins are similar to those of *Escherichia coli* and *Haemophilis influenzae*, *H. pylori* have distinct proteins, similar to other bacterial species. This discordant sequence similarity suggests lateral gene transfer. The basic mechanisms of replication, cell division and protein secretion are similar to those of *Escherichia coli* and *Haemophilis influenzae*, but important differences are noted. In particular, *H. pylori* have two specialised secretory pathways; the flagellar export pathway and an export system associated with the *cagA* PAI.

1.3.3 Cellular morphology.

H. pylori is a Gram negative microaerophilic organism that is catalase, oxidase and urease positive. It is an S-shaped or curved rod approximately 0.5-0.9 μm wide and 2-4 μm long, with a smooth external wall. In older cultures, spherical or coccoid forms predominate and may reflect a viable, more resistant and dormant form of *H. pylori*; it has not been established if coccoid forms can revert to an infectious bacillary form under appropriate conditions. The bacterium typically possesses 4 - 6 polar sheathed flagella, which consists of a terminal bulb and filaments. The filaments are composed of flagellin, a protein monomer, a hook, a cell anchored basal plate and a sheath which is continuous with the outer cell wall membrane (for review see Dick, 1990; Owen, 1998).

1.3.4 Macromolecular characteristics.

Fatty acid composition, lipopolysaccharides (LPS), genomic DNA and extrachromosomal DNA are important molecular chemotaxonomic markers of the species *H. pylori.* The major characteristic cellular fatty acids are tetradecanoic acid and *cis*-11, 12-methylene octadecanoic acid. The sole respiratory quinones present in *H. pylori* are menaquinones. The LPS is less immunogenic than that of other enteric bacteria, and the LPS is unusual in that Lewis x and y blood group antigens are expressed in up to 80% of strains. Thirty five to fifty percent of *H. pylori* strains contain plasmid DNA, with the size (1.8-40 kb) and number of plasmids varying considerably between strains. The plasmid DNA has not been associated with any biological characteristics (for review see Owen, 1998).

1.3.5 Physiological properties.

H. pylori are homogenous in terms of their cultural requirements. The bacteria are microaerophiles, growing optimally in an atmosphere of 5% oxygen with 5-10% carbon dioxide, on blood containing media such as Oxoid brain heart infusion agar and 5% horse blood agar enriched with 1% Iso Vitale X. Strains will grow over a temperature range of

33-40°C, but grow best at 37°C after a period of three to five days, with a good growth at a pH 6.9-8.0 (Goodwin *et al*, 1989).

1.3.6 Colonisation and adaptation.

H. pylori colonise only gastric type tissue. Other bacteria from the same genus, for example *H. cinaedi* and *H. fennelliae,* have been successfully isolated from the lower bowel demonstrating that the genus is not confined to the stomach as initially thought (Owen, 1998). The bacterium is highly adapted to the gastric mucosal environment and the ability of *H. pylori* to colonise gastric type epithelium despite the low pH is a unique feature. Motility has been shown to be important in colonisation and persistence, and the flagellae play a major role in the bacteria's motility (Hazell *et al*, 1986). Isogenic mutants which fail to produce flagellin have diminished ability to both colonise the mucosa of gnotobiotic piglets, compared with the wild type strains (Eaton *et al*, 1996). The flagellae and the helical morphology of the organism enables movement through the mucus, avoiding gastric peristalsis, and allowing access to the microaerobic and less acidic environment at the epithelial cell surface of the gastric mucosa. Initial colonisation involves exposure of *H. pylori* to high levels of acid, prior to penetration of the bacteria into the mucus layer. The survival of the bacterium during these extreme pH conditions is probably dependent on the urease enzyme activity and the ability of the bacterium to establish a positive internal membrane potential. The urease enzyme and the production of acid inhibiting factors have an important role in enabling *H. pylori* to survive exposure to gastric acid. The urease enzyme catalyses the breakdown of urea into carbon dioxide and ammonia molecules. The resulting alkali ammonium ions counter balance the acid produced by the gastric parietal cells and create a mildly acidic environment ideal for the bacteria's survival. Regulation of intracellular pH is vital for the survival of the bacterium in the gastric acid environment, but is poorly understood. The production of base (ammonia), cytoplasmic buffers and active transport of H^+ ions across the cytoplasmic membrane (by a H^+/K^+ ATPase proton pump) are all involved in the maintenance of

bacterial pH homeostasis and help to establish a positive inside membrane potential (McGowan *et al*, 1996).

1.4 Epidemiology and Transmission.

1.4.1 Prevalence.

H. pylori is considered to be the most prevalent pathological bacterial infection of mankind on a worldwide basis, being present in at least a third of the world's human population (Taylor *et al*, 1991). There is, however, marked geographical variation in the pattern of infection. Prevalence of infection in developing countries is higher than in developed countries (Megraud *et al*, 1989; Perez-Perez *et al*, 1994), but infection rates increase progressively with age and plateau in middle age in all geographical areas (van Zanten *et al*, 1994). In developing countries, the age of acquisition of infection is young. A seroepidemiological study carried out in several populations, showed childhood infection rates exceeding 30-40% in developing countries, and the adult infection rate was as high as 80-90% in certain populations, for example in Algeria (Megraud *et al*, 1989). The developed world has much lower rates of infection, it is uncommon for children to be colonised, and approximately 50% of adults are colonised by the fifth to tenth decade (Taylor *et al*, 1991). This progressive increase in prevalence with age is most likely due to a cohort effect, although continued acquisition throughout life is a possibility. Mendall and colleagues demonstrated that childhood factors, rather than current risk factors, strongly correlated with infection rates in adults, suggesting that the majority of seropositive adults acquired their infection as a child, and the increasing prevalence with age is due to a cohort effect (Mendall *et al*, 1992). Conversely, a three year cohort study in Canada, showed that there was a continous risk of infection throughout life, with annual seroconversion rate of 1%, and concluded a cohort effect did not solely account for the increase in prevalence with age in their study population (van Zanten *et al*, 1994). There is no convincing evidence that seroprevalance is effected by gender or smoking (Megraud *et al*, 1989), dietary intake or drinking habits (for review see International Agency for Research on Cancer, 1994).

Re-infection with *H. pylori* after eradication is controversial. There are reports of re-infection but the data varies according to geographical origin, the treatment protocol, and the time interval used to define the success of therapy (Bell et al, 1996). Recrudescence of primary infection and inadequate therapy are important considerations and probably account for a significant number of apparent re-infections. In the developed world re-infection is uncommon, particularly in adults, and is somewhere in the order of 0-1.2 % (Parsonnet, 1995). Re-infection is probably higher in developing countries, but little data exists.

1.4.2 Transmission.

It is accepted that socioeconomic status (for review see Taylor *et al*, 1991) and intrafamilial clustering are important predictors of infection, but the exact mode of transmission of *H. pylori* is still unclear. Several studies have attempted to define the route of transmission but the data is equivocal. Possible modes of transmission include direct spread (oral-oral, faeco-oral, gastro-oral), spread via animal reservoirs and spread by water.

1.4.2.1 Direct.

Non human reservoirs of *H. pylori* are rare and so it is thought that direct spread from human to human is highly likely. Oral-oral, Faeco-oral or Gastro-oral spread, have all been proposed as modes of direct transmission. Several studies have addressed the possibility of the oral cavity acting as a source of infection. Intrafamilial clustering of *H. pylori* infection has been demonstrated (Drumm *et al*, 1990). *H. pylori* DNA has been detected in saliva using polymerase chain reaction [PCR] (Birac *et al*, 1992) and has been isolated from dental plaque (Banatvala *et al*, 1993). A study of transmission of *H. pylori* from mother to child in an African population demonstrated higher seropositivity of mother and child in communities where the mother pre-masticated the food for the child at weaning (Albenque *et al*, 1990). Infection with *H. pylori* has been documented in a physician after giving mouth to mouth resuscitation to a *H. pylori* positive patient who had vomited (Figura *et al*, 1996). The organism has been isolated from faeces (Thomas *et al*, 1992) and detected in stool samples by PCR (Mapstone *et al*, 1993).

1.4.2.2 Water spread.

Many studies have looked at the possibility of water as a source of infection. *H. pylori* have been shown to survive in the metabolically active, but unculturable, coccoid form for several months in water (Bode *et al*, 1993). *H. pylori* have been successfully detected in drinking water in Peru, using PCR (Hulten *et al*, 1996). A second study on Peruvian

children showed that the type of water supply was an important factor in determining infection (Klein *et al*, 1991). Increased infection rate in the populations of the Andes has been associated with drinking and swimming in stream water (Goodman *et al*, 1996). Conversely, a number of studies have demonstrated that water sources are unlikely to be a main route of transmission for most populations (Mitchell *et al*, 1992; Teh *et al*, 1994).

1.4.2.3 Non-human reservoirs.

There is some evidence of an increased prevalence of *H. pylori* seropositivity in abattoir workers, but convincing differences in *H. pylori* seroprevalance in vegetarians have not been demonstrated (Megraud *et al*, 1989), suggesting that meat is an unlikely vehicle of transmission. *H. pylori* infection is acquired in childhood, and handling of domestic animals by young children has been hypothesized as a potential route of transmission. *H. pylori* have been isolated from domestic cats (Handt *et al*, 1994) supporting this theory.

1.5 Diagnosis of infection.

An accurate diagnosis of infection with *H. pylori* is important, especially in today's clinical setting, where antibiotic resistance is on the increase and where it is important to identify *H. pylori* associated symptomology and pathology. Diagnosis can be established by several methods, both invasive and non-invasive. Invasive techniques involve obtaining gastric biopsies during endoscopy for rapid urease test, histology and culture. Non-invasive tests are the ^{14}C and the ^{13}C urea breath test and serological tests. There is increasing interest in the use of non-invasive diagnostic methods, especially in the community and in the younger population (under 45), but variable test reliability, expense, and fear of missed pathology have so far limited this line of clinical practice.

1.5.1 Invasive tests.

1.5.1.1 Culture.

Culture has the advantage of being a highly specific means of identifying *H. pylori* infection and is essential, especially in patients with recurrent, persistent infection, for establishing antibiotic sensitivity. There are numerous problems with this technique. Ideally, due to the patchy nature of *H. pylori* colonisation, multiple biopsies are necessary. Samples can rarely be processed immediately, and thus initially require storage in a freezer. Freezing biopsies for culture introduces variables; the temperature and length of freezing influence the success of the culture yield and are important factors to be considered (Han *et al*, 1995). The culture process is time consuming, requiring up to 7 days incubation in a microaerobic environment, and contamination with micro-organisms from the oro-pharynx is a common problem, with rates of 20-25% (Piccolomini *et al*, 1997). Selective media are recommended (Fresnadillo Martinez *et al*, 1997), but the yield with these media is rarely 100%, and therefore it is often necessary to use a combination of a selective and non-selective media (Piccolomini *et al*, 1997). There is a huge variation in

the inter-and intra-laboratory culture sensitivity. All these factors mean that although culture is the most specific diagnostic test, the sensitivity of the technique can vary enormously.

1.5.1.2 Rapid urease test.

The rapid urease test utilises the powerful and abundant urease enzyme produced by *H. pylori*. A biopsy specimen is placed onto a medium containing urea and a pH indicator. The bacterial enzyme splits the urea to form ammonia, resulting in a pH change, detected by a change in the indicator colour from yellow to red. The test is quick, (allowing diagnosis at the time of the procedure), user friendly, and cheap. Generally, this test has a good sensitivity and specificity, somewhere in the order of 88% and 98-100% respectively (Malfertheiner *et al*, 1996). A number of factors may affect the rapid urease test. In particular, bacterial load (with a low load resulting in a slower indicator change and final reading), biopsy size (Laine *et al*, 1996) and patient medication. Proton pump inhibitors (PPI) inhibit the urease enzyme and alter the pattern of bacterial colonisation, with bacteria preferentially migrating to the fundus reducing bacterial density in the antrum, the usual site of the biopsy specimen for the rapid urease test (Logan *et al*, 1995).

1.5.1.3 Histology.

Histological identification of *H. pylori* and associated gastritis has a key role in diagnosis of *H. pylori* infection. This method allows identification of bacteria, the presence and severity or absence of gastritis, and confirms endoscopic diagnoses such as metaplasia and atrophy. Patchy bacterial colonisation of the mucosa may lead to false negative results. Examination of 4 biopsy specimens (two antral and two body) is recommended and minimises the inaccuracy (Dixon *et al*, 1996). Inter-observer variation is well documented, particularly in non-gastrointestinal histopathologists. The development of a universal scoring system, the Sydney System, minimises this variation (Andrew *et al*, 1994).

1.5.1.4 Polymerase Chain Reaction.

Detection of *H. pylori* specific DNA sequences by PCR has been successfully used on isolates of *H. pylori,* samples of gastric juices (Basso *et al*, 1996), faeces (Mapstone *et al*, 1993) and dental plaque (Banatvala *et al*, 1993). The gene encoding 16S rRNA has been successfully identified using direct PCR on gastric biopsy samples (Ho *et al*, 1991). It has the advantage of being extremely sensitive, with a sensitivity of over 95% and a specificity of over 85% as compared with other tests (Lin *et al*, 1996), but is a complex and time consuming technique. The presence of PCR inhibitors such as faecal material and blood can give false negatives (El-Zaatari *et al*, 1997) and contamination with cross reactivity of sequences from other bacteria result in false positives. In addition, primer selection and cycle conditions are crucial for optimizing the PCR reactions. The technique is currently used as a research tool only.

1.5.2 Non-invasive tests.

1.5.2.1 Serology.

Antibodies against *H. pylori* can be detected in human blood and serum samples by a number of serological methods, in particular ELISA (enzyme linked immunoabsorbent assay), immunoblotting and 'rapid near patient' tests.

The ELISA technique is a semi-quantitative measure of *H. pylori* infection, with a final titre reading predicting a positive or negative reading, according to a cut off value. The clinical relevance of a high positive versus a low positive titre is unclear, although the pattern of titre decline may be of use post eradication (Kosunen *et al*, 1992; Culter *et al*, 1996). An ELISA technique takes several hours to perform, is laboratory based and the performance of an ELISA can alter with day to day variation in the laboratory conditions. The use of the ELISA technique is becoming increasingly popular, and numerous commercial kits are now available on the market, both for detection of antibodies to *H. pylori* as a whole and more recently for detection of antibodies to specific bacterial proteins such as CagA. The kits vary enormously in terms of their sensitivities and specificities, and validation with adjustment for the local population is essential (Nair *et al*, 1995). A recent report by the MDA (Medical Devices Agency) (Stevens *et al*, 1996) looked at 16 different commercially available enzyme immunoassay test kits, and found that the average accuracy was 78% with the accuracy increasing in those patients under the age of 45 to 84%. There was a huge variation in the specificities and sensitivities between the tests and all were well below those quoted by the manufacturer.

Immunodetection by western blotting is also a laboratory-based technique, which requires numerous steps and is time consuming. The method is qualitative, with the presence or absence of a band, or a pattern of bands, defining a positive or negative result. Commercial kits are now available, but not in the same abundance as ELISA kits. The MDA reported

similar problems with the commercial western blotting kits as to those of commercial ELISA kits (Stevens *et al*, 1996).

The 'near patient tests' have the advantage of ease of use; they can be performed in the surgery on whole blood obtained by finger prick and read from within minutes up to 3 hours. The drop of blood is placed onto a plate coated with *H. pylori* antigen, and usually a colour change indicates a positive or negative result. Their use in diagnosis is becoming increasingly popular in the community, particularly in patients under the age of 45, with a view to avoiding upper gastrointestinal endoscopy in these patients. Like the laboratory-based techniques, there is a marked variation in the reliability of these kits and inter-observer variation can also be a problem (Stone *et al*, 1997). Caution is needed in the use of the 'near patient tests' as a means of diagnosis of *H. pylori* infection until further studies have established the reliability and cost effectiveness of such methods (for review, see Drug and Therapeutics Bulletin, 1997).

In addition to varied kit performance and accuracy, immunodetection by these methods share other common problems in their use as a diagnostic tool. The host serological response to infection persists for at least 6 months post eradication (Kosunen *et al*, 1992), and thus serology cannot distinguish current or past infection. The performance of tests are hampered by cross-reactions (Nilsson *et al*, 1997). and approximately 2% of the population fail to produce a measurable immune response (Glupczynski *et al*, 1992).

1.5.2.2 Urea Breath Tests.

The ^{13}Carbon and ^{14}Carbon urea breath test are non-invasive, relatively easy to perform, with high sensitivity and specificity rates for detecting current infection with *H. pylori*. In the ^{13}Carbon-urea breath test, the isotopically labelled urease is ingested, rapidly hydrolysed in the presence of the urease enzyme of *H. pylori* and the released labelled ^{13}Carbon dioxide is measured in the exhaled breath by mass spectrometry. Breath samples

are collected by blowing through a straw into a test tube. ^{14}Carbon-urea can be used in place of ^{13}Carbon-labeled urea and measured with a scintillation counter, but concern over the use of a radioactive isotope limits its use. The urea breath test is used most frequently in the clinical setting to assess success of eradication therapy, although there is an increasing interest in the use of this method as a means of primary diagnosis of infection in patients under 45. The main disadvantage is the expense.

1.6 Management of *H. pylori* infection.

The management of *H. pylori* infection remains controversial, particularly in the light of increasing antibiotic resistance. The literature in this field is vast and is not reviewed in detail here, but current guidelines and principles are outlined. The development of a vaccine against *H. pylori* is not discussed.

A detailed review of the management of *H. pylori* infection was drawn up to guide both the primary care physician and the specialist gastroenterologist by The European Helicobacter Pylori Study Group (EHPSG) - The Maastricht Consensus Report (EHPSG, 1997). The report outlines those infected patients in which it is mandatory to eradicate infection: PUD, low grade MALT lymphoma, gastritis with severe macroscopic or microscopic changes, and after early gastric cancer resection. It also details that eradication therapy should be given after an acute bleed, at the time of oral feeding, and that in infected patients with an NSAID-related ulcer, *H. pylori* should be eradicated and then managed on their merits. The report advises that patients with functional dyspepsia should have *H. pylori* infection eradicated, although there is no firm evidence for this. Other groups of patients in whom treatment is recommended but not mandatory is in infected patients with a family history of gastric cancer, and in patients with gastro-oesophageal reflux (GORD) when long term anti-secretory treatment is necessary.

It is accepted that successful eradication of *H. pylori* reduces ulcer recurrence over a 6-12 month period when compared to placebo and maintenance acid-suppressive drugs (as referenced by Misiewicz, 1997). However, several different treatment regimes are available, and their use varies considerably. The Maastricht Report recommends a seven day course of a PPI, twice daily, in combination with two antimicrobials; either a macrolide (clarithromycin/erythromycin) plus an imidazole (metronidazole/tinidazole), or when resistance to either of these drugs is likely then amoxycillin is substituted in its

place. Resistance to clarithromycin and metronidazole is on the increase, and in cases of treatment failure quadruple therapy should be used, with a PPI plus classic bismuth based triple therapy.

1.7 Gastroduodenal pathology.

1.7.1 Gastric inflammation.

H. pylori infection is invariably associated with active chronic (type B) gastritis in humans, with degeneration of the epithelial cells and infiltration of the gastric mucosa with acute and chronic inflammatory cells. This gastric mucosal inflammatory response is due to stimulation of the local immune system by bacterial antigens (Wyatt *et al*, 1987). Whether *H. pylori* gastritis is ever symptomatic is controversial but is a pre-requisite for PUD.

The pathogenesis of gastric inflammation is complex, but both cellular and humoral immune mechanisms are involved. At the cellular level, *H. pylori* may damage the surface epithelial cells directly or via the release of pro-inflammatory cytokines. Various antigenic proteins, such as the vacuolating cytotoxin, phospholipases and ammonia, are directly toxic to the gastric mucosa, causing cell damage and inflammation (Dixon *et al*, 1994). Cytokines (IL-6, IL8, TNFα) released from epithelial cells on exposure to bacterial antigens, such as the cytotoxin, LPS, flagellar sheath and urease, are involved in the recruitment of neutrophils and monocytes, contributing to inflammation (Crabtree *et al*, 1991; Yamaoka *et al*, 1996).

Antigen-specific immune mechanisms are important. CD4 T-helper cells are increased in *H. pylori* infected patients (Hatz *et al*, 1996). T-helper lymphocytes mediate cellular immune responses via release of IL-2 and interferon-γ, and the release of IL-4, 5 and 10 stimulate antibody production and complement activation via B-cell activation. Activation of the B-cells results in the production of IgM, secretory IgA and IgG antibodies. (For review see Bodger *et al*, 1998). Cross reactivity of antibodies against bacterial antigens with human epithelial cells may contribute to the pathogenesis of inflammation (Faller *et al*, 1997).

1.7.2 Peptic ulceration.

H. pylori is well recognised as an important aetiological factor in PUD. Excluding non-steroidal anti-inflammatory drug (NSAID) and aspirin related PUD, *H. pylori* infection is associated with up to 95% of gastroduodenal ulceration (Nomura *et al*, 1994; Kurata *et al*, 1997). Successful eradication of the bacterium gives accelerated healing and reduced ulcer relapse rates (Labenz *et al*, 1994). The exact mechanisms of PUD pathogenesis in infected individuals remain uncertain, but it is accepted that host acid regulation and bacterial strain type are important factors.

It has been demonstrated that the normal physiological acid regulatory mechanisms are disrupted in infected compared to non-infected individuals. Acid is produced by parietal cells in the gastric body and the vagal nerve and two main gastric hormones (gastrin and somatostatin) regulate acid secretion. Gastrin, which stimulates the production of acid, is released from the antral G cells, and is stimulated by gastrin-releasing peptide (GRP), cholinergic innervation, gastric distention and cytokines. Somatostatin, released from the D cells when the intragastric pH is low and by cholesystokinin, inhibits gastrin secretion. *H. pylori* can potentially colonise throughout the gastric mucosa (Dixon, 1994), but commonly predominates in the antrum, where there is a non-acid secreting epithelium. Since both the G and D cells are located in the antrum, it is not surprising that infection results in disruption of regulation of gastrin and acid release.

Initial early infection with *H. pylori* results in initial hypochlorhydria (Morris *et al*, 1987) and is thought to potentiate initial infection (McGowan *et al*, 1996). The gastric pH, however, returns to normal several months later. Chronic infection is associated with a hypergastrinaemic state and an increase in median acid output to GRP. An increase in parietal cell mass is hypothesised as a contributing mechanism, but a convincing increase in mass has not been demonstrated (for review see McColl *et al*, 1996). In infected individuals, there is an exaggerated responsiveness of gastrin to GRP, and acid to gastrin

compared to non-infected (El-Omar *et al*, 1995). In addition, the normal physiological inhibitory mechanisms from the antrum to both gastrin and parietal cells are reduced (Olbe *et al*, 1996). This results in an increase in gastrin release and impaired inhibition of acid release. On successful eradication of *H. pylori*, acid regulation returns to normal (El-Omar *et al*, 1993)and there is an increase in somatostatin cell density with a resulting fall in plasma gastrin (Tham *et al*, 1998).

In patients with DU, *H. pylori* predominantly colonise the antrum. The hypergastrinaemia seen with *H. pylori* infection is present in healthy and DU infected individuals, but differences in acid regulation have been demonstrated between these two groups. Infected individuals with DU's have an increased maximal acid secretory capacity, whereas healthy infected individuals have reduced sensitivity to gastrin (Gillen *et al*, 1998). In patients with duodenal ulcers, the increase secretory acid capacity and the impairment of inhibitory mechanisms on the G cells and parietal cells is thought to increase mucosal damage. This results in gastric metaplasia in the duodenal bulb, with subsequent colonisation and duodenal ulceration; Jang *et al* demonstrated a relationship between gastric metaplasia of the duodenum and *H. pylori* infection (Yang *et al*, 1995).

1.7.3 Gastric cancer

Gastric cancer is a common malignancy, on a world wide basis, with high mortality and although on the decline in developed countries, causes more than 750,000 deaths annually on a global basis (Murray *et al*, 1997). The aetiology of cancer is multifactorial with diet, blood group and socioeconomic status all recognised as important factors. Several prospective nested case control studies have shown an association between gastric cancer and *H. pylori* using serological data (Forman *et al*, 1991, Parsonnet *et al*, 1996; Nomura *et al*, 1991). Recently, studies have shown an association between gastric cancer and *H. pylori* infection, with a high incidence in Japan and Asia (The EUROGAST Study Group, 1993; Perez-Perez *et al*, 1997). In 1994, *H. pylori* was classed as a carcinogen (International Agency for Research on Cancer, 1994) and more recently a direct relationship between *H. pylori* infection and gastric carcinoma has been demonstrated in Mongolian gerbils (Watanabe *et al*, 1998). The association of infection with an increased risk of gastric adenocarcinoma is for both intestinal and diffuse types (Nomura *et al*, 1991) and predominately confined to non cardia carcinomas (Parsonnet *et al*, 1991). The causal relationship is not straightforward. It is only a minority of infected individuals who develop cancer, and unexplained findings such as the male predominance in gastric cancer but not in *H. pylori* infection, patients with DU never get gastric cancer, and the 'African enigma', where a low cancer rate is seen in a population with high *H. pylori* prevalence, imply that a complex aetiological relationship exists between *H. pylori* and gastric cancer.

Probably the most universally accepted hypothesis is that chronic infection leads to atrophic gastritis, which then predisposes to intestinal metaplasia and subsequently dysplasia and carcinogenesis (Correa *et al*, 1988). Intestinal metaplasia and gastric atrophy are associated with gastric cancer (Sipponen *et al*, 1983) and *H. pylori* has been associated with both intestinal metaplasia (Kuipers *et al*, 1995) and atrophic gastritis (Asaka *et al*, 1995). The pathogenic mechanism is poorly understood, complex and is multifactorial. Bacterial colonisation pattern, acid secretion capacity and dietary factors are all thought to

be important. Low acid secretors tend to have an associated pangastritis and are thought to be at risk of developing cancer. Patients at an increased risk of non-cardia gastric cancer have been shown to develop acid hyposecretion in response to infection (El-Omar *et al*, 1997). Vitamin C prevents the production of N-nitroso compounds (known carcinogens) from nitrites in the stomach (see McFarlane *et al* 1997). Levels of vitamin C are reduced in the gastric juices of *H. pylori* infected individuals (Rathbone *et al*, 1988), and has been hypothesised as a contributory factor in the development of gastric cancer in *H. pylori* infected individuals.

1.7.4 MALT Lymphoma

Mucosal associated lymphoid tissue (MALT) is not normally present in the gastric mucosa, and is acquired as part of a response to an immunological stimulus; MALT is thought to predispose to B cell MALT lymphoma. *H. pylori* infection of the gastric mucosa has been shown to be strongly associated with MALT (Witherspoon *et al*, 1991) and MALT lymphoma (Parsonnet *et al*, 1994). There is no association between *H. pylori* and non-Hodgkins lymphoma of non-gastric sites (Parsonnet *et al*, 1994). Successful eradication of *H. pylori* leading to complete regression of low grade MALT lymphoma has been demonstrated (Wotherspoon *et al*, 1993).

1.7.5 Gastro-oesophageal reflux (GORD).

The prevalence of *H. pylori* is steadily declining in the Western world. Paralleling this is an increasing incidence of GORD and adenocarcinoma of the oesophagus (Hesketh *et al*, 1989). There is concern that this effect is causal, and that *H. pylori* may in fact be protective against GORD and its consequences. However, the evidence for such a hypothesis is scanty. The interplay of *H. pylori* colonisation with host factors such as gastric acid secretion, lower oesophageal sphincter control, and gastric emptying have been implicated, but the relationships are complex, and multiple factors are involved (for

review see Labenz *et al*, 1997). It remains uncertain if *H. pylori* protects or contributes to the development of GORD and the increasing incidence of oesophageal adenocarcinoma.

1.7.6 *H. pylori* and non-gastrointestinal manifestations.

The interest in *H. pylori* infection has developed beyond the field of gastrointestinal diseases. Infection has been associated with short stature (Patel *et al*, 1995), migraine (Gasbarrini *et al*, 1997), Raynaud phenomenon (Gasbarini *et al*, 1996) and a variety of immunological diseases including Sjogren syndrome (Figura *et al*, 1994), autoimmune thyroiditis (Figura *et al*, 1996) and Henoch-Schonlein purpura (Reinauer *et al*, 1995). Of current interest, is the possible association of H. *pylori* with coronary heart disease (CHD). There have been numerous studies that have attempted to address this potential association, but the evidence is inconclusive, with confounding factors such as socioeconomic status and associated CHD risk factors contributing to conflicting results. The significance of the association remains uncertain.

1.7.6.1 Atherosclerosis and chronic infections.

Atheroma has been associated with a variety of chronic infections. Melnick and colleagues looked for evidence of CMV (cytomegalovirus) and HSV (herpes simplex virus) involvement in atherosclerosis. CMV infected cells were isolated from atherosclerotic plaques and serum antibodies to CMV associated with atherosclerosis (Melnick *et al*, 1990). An association has been demonstrated between the gram negative bacteria *Chlamydia pneumoniae (C. pneumoniae)*, in a cross sectional study looking at serum antibodies to *C. pneumoniae* (Patel *et al*, 1995). Poor dental hygiene and myocardial infarction were found to be associated in a study looking at dental sepsis as a measure of chronic bacterial infection (Mattila *et al*, 1989).

1.7.6.2 *H. pylori* and atherosclerosis.

A higher prevalence of *H. pylori* seropositivity was first noted in patients with coronary heart disease (CHD) by Mendall and colleagues (Mendall *et al*, 1994). Several studies have since been conducted, all based on serological diagnosis of *H. pylori* infection, using a variety of definitions of acute coronary syndromes. The studies have produced conflicting results. Many of the studies are small in size, and have not adjusted for confounding factors such as socioeconomic status and smoking, but five studies are considered to be significant (Mendall *et al*, 1994; Murray *et al*, 1995; Niemela *et al*, 1996; Patel *et al*, 1995; Rathbone *et al*, 1996). Two studies, one case control study looking at a total of 285 subjects (Mendall *et al*, 1994) and one cross sectional study with a total of 388 subjects (Patel *et al*, 1995), found a positive association between CHD and the presence of antibodies to *H. pylori*; each study used different criteria for diagnosis of CHD. A third study of much greater subject size, (2115 patients, 135 of whom were classified as having CHD), demonstrated a non-statistically significant trend (Murray *et al*, 1995). Two case-control studies, one with 116 cases and controls (Niemela *et al*, 1996) and the other with 342 cases and 236 controls (Rathbone *et al*, 1996) failed to demonstrate a correlation. A recent meta-analysis of 18 studies concluded that associations between *H. pylori* and vascular risk factors were largely due to chance and publication bias (Danesh *et al*, 1998).

The pathogenesis of atherosclerosis is complex and multifactorial. Several theories suggest potential pathogenic processes by which *H. pylori* infection may contribute to atherosclerosis. Antibodies to heat shock proteins have been associated with atherosclerosis, possibly with a causal role. Cross reactivity between bacterial and human heat shock proteins, resulting in autoantibody production has been postulated as a mechanism by which infection with *H. pylori* may contribute to atherosclerosis (Birnie *et al*, 1998). Inadequate folate status, resulting in increased levels of homocysteine (by inhibition of the methionine synthase reaction) has been considered as a risk factor for atherosclerosis (Markle *et al*, 1997). *H. pylori* infection is thought to reduce the

bioavailability of folate (by increasing the gastric pH and reducing ascorbic acid) and potentially the reduced folate may contribute to an increase in homocysteine. One study has demonstrated an association between hypertension and *H. pylori* infection concluding that the association between *H. pylori* and CHD is secondary to the association with hypertension (Lip, 1996). There is increasing evidence that inflammation plays an important role in the development of atherosclerosis (Ross, 1993). A correlation between *H. pylori* infection and an increase in inflammatory mediators and acute phase reagents has been demonstrated, and hypothesised as a causal relationship between *H. pylori* infection and CHD (Mendall *et al*, 1996; Niemela *et al*, 1996; Patel *et al*, 1995).

1.8 Pathogenic determinants

Pathogens share common themes in microbial pathogenicity and *H. pylori* is no exception. Bacterial adherence, toxin production, secretion and regulation, host invasion, iron scavenging and immune survival are some of the most important common factors in bacterial pathogenicity (Finlay *et al*, 1997). The ability of an organism to adhere to a host receptor is necessary for colonisation, but the number and specific role of adhesins and the host target receptors vary amongst pathogens. *H. pylori* adhesins are specific for gastric epithelial cells. Toxins are either proteins, predominantly enzymes, or non-proteinaceous, such as LPS, which are biologically active. Bacterial toxins are classified according to their structure and biochemical activity and are dependent upon tight regulatory systems, which are sensitive to multiple factors such as osmolarity, temperature and ion concentrations. Iron scavenging is essential for pathogens, but the mechanisms vary from pathogen to pathogen. Surviving the host's immune system is imperative if an organism is to survive and immune modulation, down regulation and evasion are all mechanisms employed by pathogens.

Despite these common themes of pathogenicity, *H. pylori* is a unique pathogen in that it successfully colonises the human gastric mucosa for years, avoiding the host's immune system. Infection inevitably results in chronic active gastritis, a pre-requisite for PUD, but only a minority of infected hosts go onto develop more serious gastroduodenal pathology such as peptic ulcer disease, gastric carcinoma and lymphoma. The mechanisms responsible for such a diverse pathogenicity remain unclear, but almost certainly interaction of both host and bacterial factors are important.

1.8.1 Bacterial virulence factors.

Several factors common to all strains of *H. pylori* have important roles to play in bacterial pathogenesis: specific enzymes such as urease and catalase, motility and adherence factors. However, certain strains are classed as "virulent" as defined by specific phenotypic differences. Although *H. pylori* strains show a high degree of genomic diversity, phenotypic characteristics are relatively well conserved. Two exceptions to this phenotypic homogeneity are the production of a vacuolating cytotoxin, VacA, and the production of a highly immunogenic protein, CagA, which is associated with *in vitro* cytotoxic activity, but whose function is unknown. CagA and VacA are encoded by the genes *cagA* and *vacA*. Production of *in vitro* cytotoxin activity and CagA have been implicated in the pathogenesis of gastroduodenal pathology.

Work looking at the association of these genes and proteins in relation to gastroduodenal pathology has been based on predominantly small *in vitro* studies, relying on cultured bacteria for strain typing. In addition, as more populations from a variety of geographical origins are being studied, the relationship between *vacA, cagA* and disease appears to be more complex than initially thought.

1.8.1.1 The vacuolating cytotoxin.

Vacuolating cytotoxin activity is detectable in approximately 50% of *H. pylori* strains and is encoded by the gene *vacA* (Cover *et al*, 1994). Detection of neutralising anti-cytotoxin antibodies in the serum of *H. pylori* infected individuals indicates that the cytotoxin is expressed *in vivo* (Cover *et al*, 1992). The cytotoxin precursor is about 1300 amino acids and 139-140 kDa size (Telford *et al*, 1994) and undergoes N- and C- terminal cleavage to yield a mature secreted 87-94 kDa cytotoxin (Cover *et al*, 1994). The N-terminal signal sequence (33aa) resembles other bacterial signal peptides, and is thought to be involved in the export of the cytotoxin across the bacterial cytoplasmic membrane before being cleaved (Telford *et al*, 1994). The C-terminal third of the toxin precursor is thought to

form a pore through which the toxin is secreted (Cover *et al*, 1994). Disruption of the cleaved C-terminal domain by insertional mutagenesis results in mutants with an inability to secrete detectable 90 kDa protein, indicating that the C-terminal domain is necessary for cytotoxin secretion (Schmitt *et al*, 1994). The final secretory peptide is probably cleaved further into a 37 kDa NH_2 terminal fragment and a 58 kDa COOH-terminal fragment, which remain closely associated post cleavage (Telford *et al*, 1994). Although purified denatured VacA is ~95kDa, VacA forms a ~1000kDa complex under non-denaturing conditions. This VacA oligomer consists of 12 ~90 kDa subunits assembled into large oligometric structures with complex hexameric or heptameric ring structures (Cover *et al*, 1997; Lupetti *et al*, 1996).

The toxin has been shown to be activated by acid, and the disassembly of the ring structures of the VacA oligomer is thought to be important in its activation (Cover *et al*, 1997). Once activated, the toxin is resistant to damage by acid (pH as low as 1.5) and pepsin (de Bernard *et al*, 1995). The cytotoxin induces vacuolation in mammalian cell lines (Leunk *et al*, 1988) and epithelial cell damage and mucosal ulceration on intragastric administration to mice (Telford *et al*, 1994). Isogenic *vacA* negative *H. pylori* mutants failed to induce *in vitro* cell vacuolation and failed to produce cytotoxin (Cover *et al*, 1994). The vacuoles induced by the cytotoxin have an acidic intravacuolar pH and are potentiated by weak bases (Cover *et al*, 1992). Internalisation of the cytotoxin is dependent on temperature (Garner *et al*, 1996). VacA acts on intracellular targets and is structurally similar with A-B-type bacterial toxins (for review see Cover *et al*, 1998) but it is uncertain exactly how the toxin induces vacuolation. The toxin has been shown to interfere with ion transport and the pH gradient across the cell membrane by interfering with the vacuolar ATPase proton pump of eukaryotic cells (Cover *et al*, 1993; Papini *et al*, 1993) and vacuole formation is thought to depend on stimulation of a small GTPase, rab 7 (Papini *et al*, 1997).

1.8.1.2 The *vacA* gene.

The gene encoding the vacuolating cytotoxin, *vacA,* is present in all strains, but vacuolating activity is produced on most epithelial cell lines in approximately only 50% of strains (Leunk *et al*, 1988). *vacA* was cloned and sequenced in 1994 (Cover *et al*, 1994; Telford *et al*, 1994), but subsequently, the *vacA* gene was identified to have a mosaic structure, with the existence of multiple alleles (Atherton, 1995). Atherton and colleagues initially compared the sequence of *vacA* from strains that produced the cytotoxin *in vitro* with that from strains that had no *in vitro* cytotoxin activity. They demonstrated regions of similarity and variation, with two particular regions of hyper-variability; the mid-region locus, which encodes the C-terminal portion of the final polypeptide, and the signal sequence region. PCR primers were developed to detect the mid and signal sequences, and 59 *H. pylori* strains were typed using these primers. The study demonstrated that there are two families of the mid-region (m1 and m2) and three families of the signal-sequence (s1a, s1b, and s2). All combinations of the *vacA* regions were identified except s2/m1 and it has been suggested that the s2/m1 type may be non-viable or have a selective disadvantage (Atherton *et al*, 1995). More recently, the *vacA* s2/m1 allele has been identified in a South African patient (Letley *et al*, 1998) and subtypes s1c, m2a and m2b have also been identified (van Doorn *et al,* 1998). This mosaic organisation, seen in other bacterial species such as *Neisseria gonorrhoea* and *Streptococcus pnuemoniae,* suggests genetic recombination. It is hypothesised that DNA transfer from non-*H. pylori* species occurs resulting in highly divergent regions such as the s and m regions of *vacA*, which subsequently transfer between *H. pylori* strains (Atherton 1995, *et al*; Garner *et al*, 1995).

The work in the USA (Atherton *et al*, 1995) demonstrated important associations between the *vacA* genotypes, levels of *in vitro* toxin production and disease type. The allele s1 was associated with *in vitro* toxin production, whereas the type s2 consistently failed to produce toxin activity. Within the m1 isolates, s1a produced greater levels of toxin than s1b. The mid-region type was closely associated with *in vitro* toxin activity (m1> m2). *In*

vitro cytotoxin production was greatest in the s1/m1 strains, then s1/m2 and lowest in s2/m2. There was a significant correlation between the type s1 and a second gene, the cytotoxin associated gene, *cagA*. Characterisation of *vacA* subtype to the presence of PUD, demonstrated that type s1 strains were associated with PUD, with no significant difference between s1a and s1b. The *vacA* m type was not associated with the presence of PUD. Atherton and colleagues hypothesised that type s1 may be a marker for unidentified factors that influence the function of the cytotoxin. Alternately, differences in the sequences result in different cleavage points of the cytotoxin precursor and therefore different mature protein functions.

Further analysis of the *vacA* subgenotypes has suggested an association between the s1 type and bacterial density *in vivo* (Atherton *et al*, 1996) and the s1 type and enhanced gastric inflammation and duodenal ulceration (Atherton *et al*, 1997).

1.8.1.3 The cytotoxin associated gene, *cagA*.

The *cagA* gene is present in approximately 30-90% of strains, according to different study populations and geographical areas (Perez-Perez *et al*, 1997), and if present is virtually always expressed. *cagA* encodes a highly immunogenic 120-140 kDa protein, CagA, (Covacci *et al*, 1993; Tummuru *et al*, 1993), the function of which is unknown. The size variability of CagA is reportedly due to the presence of repeating sequences within the gene, the 'variable region', located in the 3` region of the gene (Covacci *et al*, 1993). *cagA* expression is closely associated with *in vitro* cytotoxic activity (Atherton *et al*, 1995; Cover *et al*, 1990), although *cagA* and *vacA* are not physically linked on the chromosome. Isolates lacking *cagA* do not produce toxin *in vitro* (Covacci *et al*, 1993; Tummuru *et al*, 1993), but insertional mutagenesis of *cagA* fails to disrupt toxin expression (Tummuru *et al*, 1994).

Initial identification of CagA and its importance was first noted serologically. Mucosal recognition of the *H. pylori* 120kDa protein was associated with PUD and active gastritis (Crabtree *et al*, 1991), and further studies demonstrated a link between cytotoxin positive strains and CagA (Crabtree *et al*, 1992; Covacci *et al*, 1993). *cagA* is now recognised as a marker of enhanced virulence, with several groups demonstrating a strong association with PUD (Crabtree *et al*, 1991; Covacci *et al*, 1993; Cover *et al*, 1995) and gastric cancer (Blaser *et al*, 1995; Parsonnet *et al*, 1997).

The function of *cagA* is unknown, but is a marker for a large segment of DNA which has been termed the '*cag* -pathogenicity island ' (Censini *et al*, 1996). This ' island ' is a 40-Kb foreign DNA insertion, of uncertain evolutionary origin, flanked by 31 bp direct repeats, with a distinct G+C abundance of 35%. The G+C content is different from the 38-45% G+C values typical of chromosomal sequences of *H. pylori* and is similar to that found in a plasmid of *H. pylori* strains. The presence of the direct repeats and the G+C content of the *cag* region suggest that the region has been acquired from an external source, most likely a bacteriophage or plasmid, by vertical transmission and recombination (Censini *et al*, 1996). *cagA* is associated with the induction of several pro-inflammatory cytokines (inter-leukin [IL] 8 in gastric epithelial cells and IL-1, 6, 8, 10, and tumour necrosis factor alpha [TNFα]) in gastric biopsies (Peek *et al*, 1995; Yamaoka *et al*, 1997), but isogenic *cagA* negative mutants retain the ability to secrete IL-8 (Crabtree *et al*, 1995). Two other genes have subsequently been identified, *picA* and *picB,* which are encoded in the *cag* region and are involved in the induction of IL-8 expression (Tummuru *et al*, 1995). Disruption of other genes in the *cag* region (*cagE, cagG, cagH, cagI, cagL* and *cagM* and other genes in the left half of the *cagA* PAI) reduces the ability of *H. pylori* to IL-8 secretion from gastric epithelial cells (Censini *et al*, 1996; Li et al, 1999). Several genes in the *cag* region have homologies to genes from other bacterial species that are involved in protein delivery systems and DNA transfer, implying that the *cag* region is involved in gene transfer (Akopyants *et al*, 1998). An example of gene transfer has been recently demonstrated;

cagA has been shown to encode a molecular syringe through which CagA is delivered into the host epithelial cell. Tyrosine phosphorylation of CagA occurs resulting in active cytoskeleton rearrangement (Segal *et al*, 1999; Asahi *et al*, 2000; Odenbreit *et al*, 2000; Stein *et al*, 2000).

1.8.1.4 Gastric inflammation, *vacA* and *cagA*.

Recent work has demonstrated a relationship between extent of gastric inflammation and strain type. *cagA* positive strains are associated with a greater inflammatory infiltrate when compared to *cagA* negative strains (Peek *et al*, 1995) and several groups have demonstrated a correlation between *cagA* and increased IL8 levels *in vitro* (Crabtree *et al*, 1995) and *in vivo* (Peek *et al*, 1995). Atherton and colleagues have shown that the type *vacA* s1 is associated with increased inflammation (Atherton *et al*, 1997). The effects of CagA and the cytotoxin have been studied in a mouse model, and a relationship between inflammation and the expression of CagA demonstrated, and an association between cytotoxin phenotype and epithelial damage, but not inflammation (Ghiara *et al*, 1995). A recent study looked at *cagA, vacA,* severity and pattern of gastritis and concluded that the bacterial factors *cagA* and *vacA* were important in determining the severity of inflammation but not the pattern (Warburton *et al*, 1998).

1.8.1.5 *vacA*, *cagA,* acid regulation and PUD.

A study looking at somatostatin concentrations, *vacA* type and *cagA* status demonstrated lower somatostatin concentrations with the s1 type and *cagA* positive strains (Queiroz *et al*, 1996). The GRP stimulated serum gastrin concentration is increased in infected individuals with antibodies to CagA, although an expected increase in acid output was not seen (McColl *et al*, 1997). Several groups have demonstrated a strong correlation between gastroduodenal ulceration, presence of *cagA* (Covacci *et al*, 1993; Cover *et al*, 1995; Peek *et al*, 1995) and cytotoxin producing strains (*vacA* s1) (Atherton *et al*, 1995; Cover *et al*, 1990).

1.8.1.6 Gastric cancer, *vacA* and *cagA*.

An association between *cagA* and gastric cancer has been demonstrated (Blaser *et al*, 1995; Parsonnet *et al*, 1997), but this finding is not universal, particularly in populations where the rate of *cagA* positive strains is high (Mitchell *et al*, 1996; Maeda *et al*, 1997). A recent study suggests a relation between *cagA* and vitamin C levels (Zhang *et al*, 1998). Similarly, in studies looking at *vacA* subtypes and cancer the findings conflict (Ito *et al*, 1997: Matsukura *et al*, 1997; Rudi *et al*, 1997). Cytotoxin producing strains (Fox *et al*, 1992) and *cagA* (Beales *et al*, 1996) have been shown to correlate with atrophic gastritis.

1.8.1.7 MALT lymphoma and *cagA*.

The association of *cagA* positive strains of *H. pylori* and the B cell MALT lymphoma is disputed. Eck and colleagues studied the sera of 68 MALT lymphoma patients and found a strong (95.5%) correlation with anti-CagA antibodies compared with controls (Eck *et al*, 1997), but typing of strains for the *cagA* gene in a series of 12 patients with MALT lymphoma failed to find a significant association (De Jong *et al*, 1996).

1.8.1.8 GORD and *cagA*

There is an increasing interest in the relationship between *H. pylori* and GORD. More recently, it has been shown that *cagA* positive strains of *H. pylori* may play a protective role against complications of GORD, such as Barrett's oesophagus and adenocarcinoma of the oesophagus (Vicari *et al*, 1998).

1.8.1.9 *cagA* and atherosclerosis.

Inflammatory mediators have been shown to be important in the pathogenesis of atherosclerosis (Ross, 1993) and several studies suggest a relation between acute phase reagents, inflammatory mediators and *H. pylori* (Mendall *et al*, 1995; Niemela *et al*, 1996; Patel *et al*, 1995). The association of gastric mucosa pro-inflammatory cytokines (IL-6 and IL-8, TNF α) and *H. pylori* infection is well established (Crabtree *et al*, 1991; Crabtree *et al*, 1994). More specifically, *cagA* positive strains are associated with the induction of various cytokines and an enhanced inflammatory response (Peek *et al*, 1995; Yamaoka *et al*, 1997). Potentially, *cagA* positive strains may play an important role in the pathogenesis of CHD via enhanced induction of inflammatory mediators.

Only one study to date has been published looking at the potential association of *cagA* positive strains with CHD (Pasceri *et al*, 1998). The study measured serum anti-CagA antibodies in a series of 88 cases and 88 controls in an Italian population, and correlated the findings with the presence of CHD. They demonstrated a positive association between *H. pylori* and CHD, and more interestingly anti-CagA antibodies and CHD. Pasceri and colleagues commented that the findings support the theory of an inflammatory mediated mechanism in the development of atherosclerosis. Further studies are needed to clarify these findings, and in particular, populations from different geographical areas need to be studied.

1.8.1.10 Other bacterial virulence factors.

1.8.1.10.1 Urease.

The urease enzyme of *H. pylori* is an essential, potent multisubunit structure, which is in high abundance. It is 60,000 - 65,000 kDa in size, and is associated with the outer membrane and flagella. The enzyme is encoded by two genes, *ureA* and *ureB* (Clayton *et al*, 1993), it is a nickel metalloenzyme (Hu *et al*, 1993) and is produced by all strains of *H. pylori* although at differing levels. Urease acts both internally in the cytoplasm (Scott *et al*, 1998) and is actively transported out of the cell where it acts extracellularly and hydrolyses both endogenous and exogenous urea to ammonium and carbamate (as referenced by Scott *et al*). The latter is then converted to soluble carbon dioxide. The true function of urease remains uncertain, but it is thought that urease produces an acid buffer in the form of ammonia, enabling the bacteria to survive the acidic gastric conditions for long enough to allow penetration of the epithelial mucus layer, where the pH is higher. In addition to pH regulation, it is accepted that urease is important in facilitating colonisation. Eaton and colleagues demonstrated that urease-negative mutant strains of *H. pylori* failed to colonise gnotobiotic piglets, and that it was the enzyme activity, not the protein, that was the important colonising factor (Eaton *et al*, 1994). Urease provides nutritional nitrogen from ammonia for amino acid synthesis, but this is thought to be a trait which *H. pylori* has evolved as a means of adaptation, rather than an essential function (for review see Lee, 1993).

It has been suggested that the production of ammonium ions from urease activity directly contributes to gastric mucosal damage. Megraud and colleagues showed that wildtype *H. pylori* produce vacuoles in Hep2 cells, whereas urease negative mutants do not (Megraud *et al*, 1992). In a study of rats, antral atrophy resulted in those rats whose drinking water was replaced by 0.01% ammonia, compared with no atrophy in those with no change to the drinking water (Tsujii *et al*, 1993). Conversely, a study looking at mucosal damage in *H.*

pylori positive uraemic patients compared to *H. pylori* positive non-uraemic patients showed similar levels of severity of gastritis although the uraemic patients had much higher gastric juice ammonium concentration (El Nujumi *et al*, 1992).

1.8.1.10.2 Catalase.

The enzyme catalase protects *H. pylori* against the damaging effects of oxygen metabolites. *H. pylori* have a very high catalase activity, and it is suggested that its protective role contributes to the virulence of the bacteria (Hazell *et al*, 1991).

1.8.1.10.3 Motility.

The importance of motility has been demonstrated in many gut pathogens, for example *Campylobacter jejuni* and *Salmonella spp. H. pylori* use polar sheathed flagella to propel themselves in the gastric environment and access the less hostile and less acidic submucosa. This motility is thought to enable *H. pylori* to selectively colonise the viscous mucus environment and non-motile variants of *H. pylori* have been shown to have reduced ability to effectively colonise gastric epithelium in comparison with the parent strain (Eaton *et al*, 1996).

1.8.1.10.4 Adhesins.

Although adherence factors are not directly pathogenic, adherence of *H. pylori* to the gastric mucosa is thought to promote bacterial colonisation of gastric surfaces. The identity of these specific adhesins is unclear. Both lipopolysaccharide and a lectin-like adhesin are involved in the binding of *H. pylori* to the basement membrane protein laminin (Valkonen *et al*, 1994), but several other candidates have been described.

H. pylori produce sheathed flagella, whose central role is motility. Flagella have been described as adhesins for enteric *Campylobacters* (Newell *et al*, 1985) and their role as adhesins in *H. pylori* needs to be clarified. It remains uncertain whether this effect is solely

due to the ability of the bacterium to stay closely associated with the mucosal surface through motility or whether flagellin proteins are involved in adhesion to mucosal surface.

The urease enzyme is a surface enzyme, which has direct cytopathic effects and possible adherence properties. Work by Fauchere and colleagues showed that strains with the best binding to HeLa cell membranes also had the highest urease activity (Fauchere *et al*, 1990). In contrast, Clyne and colleagues showed that urease negative mutants bound to Kato III cells and primary epithelial cells as successfully as the parent strain (Clyne *et al*, 1993).

H. pylori cause agglutination of erythrocytes, and specific haemagglutinates have been described as adhesins. A haemaglutinin which binds to a specific neuraminyl lactose-containing membrane receptor has been described (Evans *et al*, 1988), but others have failed to demonstrate a correlation between haemaglutinins and ability to adhere to cell lines *in vitro* (Clyne *et al*, 1993).

Several surface proteins have been identified as potential adhesins but the evidence is conflicting and inconclusive. In particular, a 63kDa protein and a 19.6 kDa protein have been described as potential candidates (For review see Sherman PM 1993).

Lipopolysaccharide (LPS) is a major constituent of Gram-negative bacterial cell walls. *H. pylori* bind to collagen and to laminin, and purified LPS has been shown in one study to inhibit binding to laminin (Slomiany *et al*, 1991) suggesting that this bacterial surface molecule may be involved in the adhesion process. A recent study, using monoclonal antibodies that inhibit adhesion of *H. pylori* to human gastric cancer cells, showed that the monoclonal antibodies recognised LPS, implicating LPS as an adhesin (Osaki *et al*, 1998).

The blood group antigen binding adhesin (BabA) has been identified as an adhesin for the Lewis antigen on gastric epithelial cells (Gerhard *et al*, 1999). Gerhard showed that there

was a correlation between the gene *babA2* with *cagA, vacA s1* and the presence of PUD and gastric cancer. However the pathogenic relevance of this adhesin remains uncertain.

1.8.1.10.5 The *iceA* gene

The gene *ice A* (induced by contact with epithelium) has recently been discovered (Peek *et al*, 1996), and has two main allelic variations: *iceA* 1 and *iceA* 2. The function of *iceA* is unknown, but *iceA* 1 is associated with the presence of PUD, and is thought to be a marker for PUD, independent of *cagA* and *vacA* (van Doorn *et al*, 1998).

1.8.2 Host response.

It is likely that *H. pylori* survives in the human gastric mucosa for years. This successful co-existence between bacterium and host suggests that *H. pylori* is not a true parasite. Most recent theories propose that in fact the bacterium should be classified as amphibiotic, where an organism may be beneficial or disease causing depending on the host and microbe population biology (Blaser, 1997). The host response to infection is increasingly recognised as an important determinant of disease outcome, although lack of an ideal animal model that mimics human disease limits the ability to study host responses (Lee, 1998).

1.8.2.1 Acid secretion.

The intragastric acidic environment is very likely to be an important factor in determining disease patterns in patients with *H. pylori* infection. Acid secretion varies in a normal distribution, and is genetically and environmentally determined. Disease outcome of infection with *H. pylori* is thought in part to depend on the acid secretory capacity of the host; high acid secretors are predisposed to duodenal ulcers and low secretors to gastric atrophy, which predisposes to gastric cancer (Graham *et al*, 1997). The determining factors of the host's acid secretory capacity are complex, but the pattern of bacterial colonisation is thought to be important, with predominantly antral gastritis resulting in an hyper-secretory state and pangastritis resulting in hyposecretors. Host factors affecting the cytokine IL-1-beta (an inhibitor of gastric acid secretion) may determine disease outcome; El-Omar and colleagues have demonstrated an association between interleukin-1 gene cluster polymorphism (thought to enhance IL-1beta production) and an increase in *H. pylori* induced hypochlorhydria and gastric cancer (El-Omar et al, 2000). Childhood nutritional status has also been shown to be important in determining acid secretory capacity, with malnutrition resulting in low acid secretion (Gracey *et al*, 1997).

1.8.2.2 Immune response.

The host immune response, both cellular and humoral, is important in the pathogenesis of disease in individuals infected with *H. pylori.* Heterogeneity of the immunoregulatory genes and the host response to pro-inflammatory mediators are both of relevance. A number of important antigens are produced by *H. pylori* that are involved in indirectly inducing the host's immune response; urease, the vacuolating cytotoxin, lipopolysaccharides and heat shock proteins. These bacterial antigens induce T-helper cells, epithelial cells and macrophages, and stimulate the release of pro-inflammatory cytokines (TNF-α, interferon-γ [IF-γ], IL-8). Released cytokines induce inflammation by direct toxicity, chemoattraction of leukocytes and other inflammatory cells (Telford *et al*, 1997) and possibly via enhanced acid secretion secondary to cytokine induced hypergastinaemia (Playford, 1996). Interleukins, in particular IL-6, stimulate the B-cells, with production of IgM, secretory IgA and IgG.

Immune elimination of the bacteria consists mainly of complement-dependent phagocytosis, stimulated by IgG antibodies. In addition, secretory IgA limits bacterial adhesion (Dixon, 1994). However, a characteristic feature of the organism is its ability to evade the immune system. Several mechanisms are involved in this dampening of the host immune response. The LPS of *H. pylori* is less immunogenic than that of other enteric bacteria, ammonia production inhibits phagocytosis, and bacterial surface haemagglutinins may delay adhesion and ingestion of *H. pylori* (for review see Wadstrom, 1995).

The development of small gastric lymphoid aggregates (Wyatt *et al*, 1988) and the association of a low-grade B cell gastric lymphoma of the mucosal associated lymphoid tissue (MALT) with *H. pylori* infection (Wotherspoon *et al*, 1991) emphasise the potential importance of the immune response in the final outcome of infection.

1.8.2.3 Autoimmunity and mimicry.

H. pylori induced autoantibodies may cross react with host epithelial components, and has been postulated as a potential mechanism for *H. pylori* induced mucosal damage. Faller and colleagues are amongst several groups who have demonstrated an association between the presence of these antigastric autoantibodies, severity body gastritis and gastric atrophy (Faller *et al*, 1997). The Lewis x/y blood group antigens have been identified on the LPS of most *H. pylori* strains, and are also present on gastric epithelial cells. This antigenic mimicry may result in immune tolerance against antigens of the pathogen or the production of autoantibodies, the presence of which have been shown to correlate with the extent of *H. pylori* induced gastric body atrophy in animals (as referenced by Appelmelk *et al*, 1998). However, the autoimmune mechanism in humans is more complex, and it is uncertain whether molecular mimicry or inflammation itself results in anti-gastric autoreactivity (Faller *et al*, 1997).

1.9 Aims and rationale.

H. pylori infection is one of the commonest bacterial infections world wide and the commonest gut pathogen. Infection inevitably results in an asymptomatic chronic active gastritis, but a minority of infected individuals will go onto develop more serious gastroduodenal pathology. The pathogenesis of *H. pylori* associated disease is complex, multi-factorial and remains unclear, but bacterial, host and environmental factors are the major contributors to the diverse clinical outcome of infection with *H. pylori.*

Attention has been focused on bacterial pathogenic determinants and their potential role in the development of *H. pylori*-associated disease. Two genes, *vacA* and *cagA,* and their gene products, the vacuolating cytotoxin, and CagA, have been identified as potentially important virulent factors. The vacuolating cytotoxin is the only protein toxin of *H. pylori* identified so far. Although these factors (*vacA* and *cagA*) may potentially identify virulent strains of *H. pylori,* the work is predominantly *in vitro*, and the clinical importance of *vacA* and *cagA* is based on a narrow geographical study group (the USA). The relevance of these bacterial factors and their relationship with gastroduodenal pathology needs to be clarified and defined in different study populations.

Theoretically, screening for virulent strains would allow targeting of the management of patients at greatest risk of developing *H. pylori* associated disease. Thus, it is essential that the role and significance of *vacA* and *cagA* in the pathogenesis of gastric inflammation and disease is established, in order to ascertain if these bacterial factors are to have any future clinical applications.

The current work started in 1996, with the aim of studying bacterial heterogeneity and its role in the clinical and pathological diversity of *H. pylori* infection.

The specific aims of this thesis are: -

1. to establish if it is possible to successfully type the *cagA* gene and the *vacA* alleles directly from biopsy DNA, without the need for culture.
2. to establish if *in vitro* strain types are representative of *in vivo*.
3. to clarify the relationship between microscopic and macroscopic gastroduodenal pathology, *cagA* status and *vacA* subtypes in a UK study group.
4. to see if the relationship between myocardial infarction and *H. pylori* infection can be further clarified in terms of the type of infecting strain.
5. to look at the size of the CagA protein, macroscopic and microscopic pathology.
6. to detail serological response in infected individuals to CagA and VacA.

1.10 Study design.

1.10.1 Clinical studies. The relationship of *H. pylori* genotypes with gastroduodenal microscopic and macroscopic pathology was studied in a group of dyspeptic patients from outpatient clinics and inpatient wards. Macroscopic pathology was defined by gastroscopy.

1.10.2 Typing techniques. The *vacA* s and m types and the *cagA* status were typed from biopsy and isolate DNA using PCR.

1.10.3 Serological studies. Serological responses were studied using western blotting and ELISA assays. The assays were clinically evaluated for use on the study population.

1.10.4 Isolate studies. Isolates were cultured, and the protein profile studied by staining proteins with a silver staining technique. Isolate antigens were identified using a mouse monoclonal anti-CagA antibody, and serum from a CagA positive infected individual.

Chapter 2

Materials and Methods

2.1 Introduction.

The patient studies were carried out with the approval of Leicester Royal Infirmary Ethical Committee. Manufacturers and suppliers of reagants are listed in Appendix II. The abbreviations used in the text are listed in Appendix III and details of buffer reagents and solutions are given in Appendix I. Patient information and consent sheet are outlined in Appendix IV. Appendix V details all the supplementary data for Chapters 3, 4, 5, and 6.

2.2 Subjects and clinical assessment.

2.2.1 Dyspeptic patient selection

Patients were selected from routine attenders to the endoscopy suite, who were being investigated for symptoms of dyspepsia, and in-patients requiring upper gastrointestinal endoscopy. Each patient was provided with a patient information and a consent sheet which were completed accordingly. Patients were defined as having PUD if active ulceration was noted at the time of endoscopy.

The patients were selected according to the exclusion and inclusion criteria.

Inclusion criteria:

Male and/or female patients >17 years of age.

Symptoms of dyspepsia.

Exclusion criteria:

On NSAIDs within the previous 7 days.

On proton pump inhibitors within the previous 7 days.

Previous gastric surgery.

Previous *Helicobacter pylori* eradication therapy.

2.2.2 Coronary heart disease and anti-CagA antibody study.

Cases and controls used in the IHD study are described in detail in chapter 5.

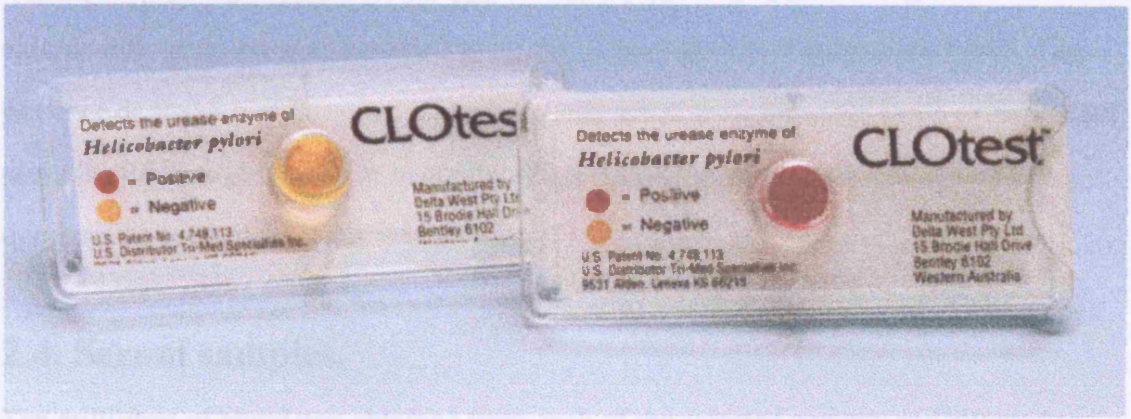
2.2.3 Endoscopy.

Patients attending for routine upper GI endoscopy were consented. The endoscopy was carried out by one of a number of experienced endoscopists in the Gastroenterology Department of the Leicester Royal Infirmary, using an Olympus (XQ200 and 230 series) video gastroduodenoscope. Each scope was cleaned between each patient following the BSG guidelines (Cowan *et al*, 1993). All patient's throats were sprayed with xylocaine 1% (Astra). Sedation was given using intra-venous midazolam (2.5mg-5mg) if requested by the patient. Macroscopic appearance of the oesophagus, stomach and duodenum were noted at the time of the proceedure. Endoscopic biopsies were taken routinely from the antrum for histology and a rapid urease test. Abnormalities including suspicious lesions, were biopsied for histology at the time of the endoscopy. Additional samples were obtained from the following areas :

- 1 biopsy from the posterior wall of the duodenal cap
- 2 biopsies from the antrum (approx. 5cm from the pylorus)
- 1 biopsy from the antral-body junction, greater curve
- 1 biopsy from the greater curve of mid-body
- 1 biopsy from the fundus

The biopsies were taken using Olympus non-spiked reusable biopsy forceps, which was passed through the forcep channel on the gastroscope and mucosa obtained on closure of the forceps. Biopsies for histology were placed immediately in 10% buffered saline. Samples for culture and PCR were placed into a 2ml tube containing transport medium (brucella broth containing 10% (v/v) glycerol), using a sterile orange needle. The tubes were then stored at -20°C. The antral biopsy sample for the rapid urease test, the CLO test (Campylobacter-Like-Organism) [Delta West], was placed onto the test medium to detect the presence of *H. pylori*. The CLO test was left for up to 24 hours. A colour change from orange to magenta confirms the presence of the *H. pylori* with approximately a 98-100% sensitivity (Malfertheiner *et al*, 1996).

Figure 2.1 Commercially available CLO test showing the colour change from negative to positive in the presence of the urease enzyme of *H. pylori.*



2.3 Histological assessment.

Antral biopsy samples were placed on filter paper and fixed in 10% formalin. Tissue was processed within 24 hours in the routine histology laboratory. Sections were stained with H&E and with a modified Giemsa stain. The sections were examined by a single experienced Consultant Histopathologist, Dr. K.P.West, who was blinded to the CLO result and infecting *H. pylori* strain type. Extent of bacterial density and grade of acute and chronic inflammation were assessed using the Sydney System (Dixon *et al*, 1996). The presence or absence of intestinal metaplasia was noted using the H&E stain, or facilitated using the PAS reagant alone or in combination with alcian blue at pH 2.5. Grade of dysplasia or overt malignancy was noted.

2.4. Serum samples.

Ten millilitres of blood was obtained from each of the selected patients and allowed to clot. The serum was removed using a sterile pipette into a sterile eppendorf, centrifuged for 30s at 10,000g in a microcentrifuge (MicroCentauer) and stored at -20°C. All samples were frozen within 24 hours of being taken.

2.5 Bacteria.

The strains used in this study were isolated in the laboratory as described below. The well characterised *cagA* positive strain *H. pylori* NCTC 11637 was obtained from the National Collection of Type Cultures.

2.5.1 *H. pylori* culture.

Gastric antrum biopsy samples for culture were placed in tubes containing transport media (brucella broth containing 10% (v/v) glycerol) and stored at -70°C until processing. Biopsies were mashed with a sterile scapel in a sterile petri dish and inoculated onto *Helicobacter* selective agar (Dent & McNulty agar containing selective supplement, Oxoid) and chocolate agar. The plates were incubated at 37°C in a microaerobic atmosphere (Campylobacter System Gas Generating kit, Oxoid Ltd) for up to 7 days. All isolates were subcultured onto chocolate agar using single colonies from the primary isolation plate where possible. Where single discrete colonies could not be obtained, a sweep of growth was taken and subcultured for purity. Plates were incubated as before, at 37°C for 48h and isolates harvested into brucella broth containing 10% (v/v) glycerol and stored at -70°C. Organisms were identified as *H. plyori* by Gram stain, and by oxidase, catalase and rapid urease test.

Figure 2.2 *H. pylori* growth on a chocolate agar plate.

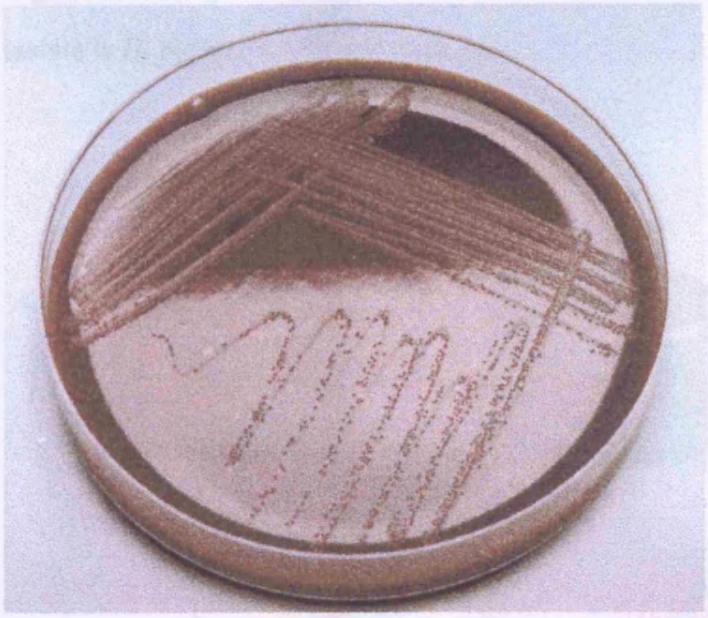


Figure 2.3 Close up of *H. pylori* colonies.

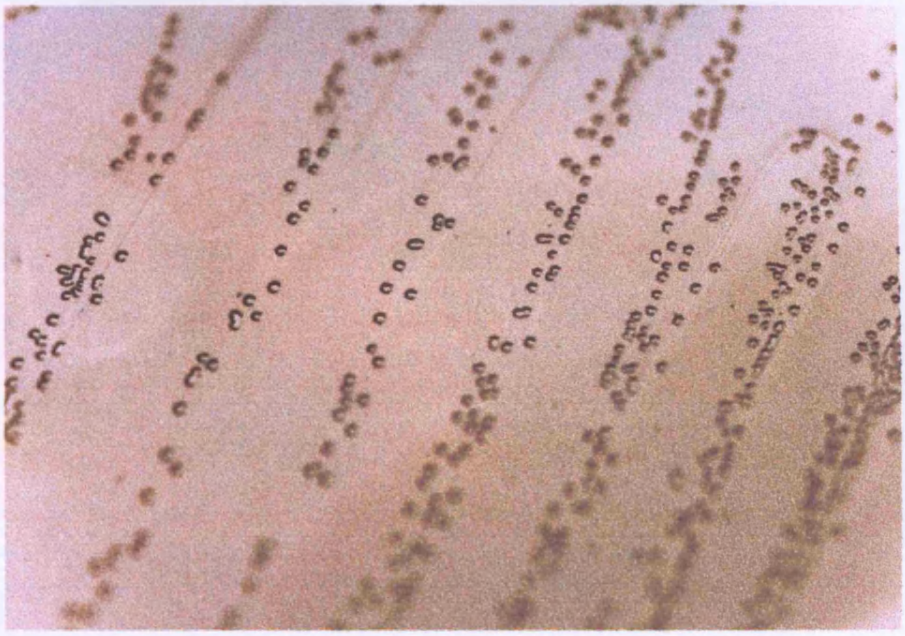


Figure 2.4 A negative and positive result of the urease test, used to confirm the cultured isolate is *H. pylori*.

2.5.2 Antigen preparation.

Clinical isolates of *H. pylori* were cultured from antral biopsy specimens as described above, harvested into small volumes of Brucella broth containing 10% (v/v) glycerol and kept at -70°C. The isolates were then thawed, and spread plated onto chocolate agar and grown at 37°C for 48 h under microaerobic conditions. Bacteria from a single plate were harvested with a sterile swab into 500µl of sterile PBS and vortexed vigorously. The bacteria were then centifuged at high speed in a microcentrifuge to remove cells and debris and the supernatant retained as the PBS wash antigen for use in SDS-PAGE and immunoblotting.

2.6 DNA preparation.

2.6.1 DNA preparation from bacteria.

Bacterial chromosomal DNA was prepared from isolates spread plated onto chocolate agar and grown in a microaerobic atmosphere at 37°C for 48h. Bacteria were harvested into 500μl of sterile phosphate buffered saline (PBS pH 7.4) and washed once. Cells were pelleted by centrifugation (10,000g, 5min) and resuspended in 200μl of digestion buffer containing 100μg/ml lysozyme [Sigma]. Samples were incubated at room temperature for 15min, and SDS added to a final concentration of 1% (v/v). Bacterial cell lysis was completed by heating to 65°C, before addition of proteinase K [Sigma] to 25μg/ml. Samples were incubated at 50°C for 2h. Protein was removed by phenol/chloroform extraction and DNA recovered by ethanol precipitation and resuspended in 100μl of sterile 18 megohm water [Sigma].

2.6.2 DNA preparation from biopsy tissue.

The four gastric and one duodenal tissue samples were placed in transport media tubes, and stored at -20°C until processed. The biopsies were individually placed in a sterile petri dish and mashed with a sterile scalpel . The tissue sample was resuspended in 300μl of digestion buffer containing 25μg/ml of proteinase K (Sigma) and incubated at 37°C overnight. Protein was removed by phenol/chloroform extraction and DNA isolated by ethanol precipitation.

2.6.3 Phenol/chloroform extraction.

An equal volume of phenol: chloroform: isoamyl alcohol mix (25:24:1; Sigma) was added to the digested tissue sample or lysed bacterial preparation and the sample mixed gently by inversion. The samples were centrifuged at high speed in a microcentrifuge. The aqueous layer (top layer) was removed and transferred to a fresh 1.5ml tube whilst taking care not to disturb the interface. Where necessary, the phenol extraction was repeated to remove all the precipitated protein from the aqueous layer. Residual phenol was removed

from the samples by a chloroform extraction. An equal volume of chloroform: isoamyl alcohol mix (24:1; Sigma) was added and the samples mixed by inversion, and centrifuged at high speed in a microcentrifuge for 5 min. The aqueous layer containing the nucleic acids was removed and the DNA recovered by ethanol precipitation.

2.6.4 Ethanol precipitation.

DNA was precipitated by the addition of 1/10 th volume of 3M sodium acetate and 3 volumes of ice cold absolute ethanol and the samples left at -20 °C for at least 1h. The DNA was pelleted by high speed centrifugation in a microfuge for 10min and the supernate was discarded. The pellet was allowed to dry in air, and then resuspended in 50μl of sterile 18 megohm water (Sigma).

2.7 The Polymerase Chain Reaction.

2.7.1 Primers.

Detection of the *cagA* gene and the *vacA* genotypes was carried out using primers and protocols from the literature (Atherton *et al*, 1995; Tummuru *et al*, 1993). The primers used in the study are described in Table 2.1. The primers were sythesised at the Protein and Nucleic Acid laboratory, Leicester University.

Table 2.1 PCR primers used in typing of *cagA* and *vacA* alleles (modified from Atherton *et al*, 1995)

Target	Primer sequence	Fragment size	Reference
cagA	F:5`GATAACAGGCAAGCTTTTGAGG 3` R:5`CTGCAAAAGATTGTTTGGCAGA3`	349bp	Tummuru, 1993
vacA m1	F:5`GGTCAAAATGCGGTCATGG3` R:5' CCATTGGTACCTGTAGAAAC 3'	290 bp	Atherton, 1995
vacA m2	F:5' GGAGCCCCAGGAAACATTG 3' R:5' CATAACTAGCGCCTTGCAC 3'	352 bp	Atherton, 1995
vacA s1a*	F:5' GTCAGCATCACACCGCAAC 3'	190 bp	Atherton, 1995
vacA s1b*	F:5' AGCGCCATACCGCAAGAG 3'	187 bp	Atherton, 1995
vacA s2*	F:5' GCTAACACGCCAAATGATCC 3'	199bp	Atherton, 1995
US	F:5`ATGGAAATACAACAAACACAC 3` *R:5`CTGCTTGAATGCGCCAAAC 3`	s1 259 bp s2 286 bp	Atherton 1995

F=forward primer sequence, R=reverse primer sequence.

*F primers for s1a, s1b, and s2 were used with the R primer from the US primers.

2.7.2 Detection of the *cagA* gene.

The *cagA* gene was detected using the primers F1 and B1 (Tummuru *et al*, 1993) to amplify a 349bp fragment from the middle of the gene.

For detection of the gene in bacterial chromosomal DNA preparations, about 500ng of DNA in a volume of 1µl was used, for a reaction mix of 50µl. The reaction mix constituents are listed in the appendix A1. The primers were used at a concentration of 10pmol. BioTaq ™ DNA polymerase was obtained from BioLine and 2 units used per reaction tube.

The amplification protocol used was as described by Atherton and co-workers (Atherton *et al*, 1995) using 30 cycles of 94°C for 30s, 60°C and 72°C for 75s. For detection of the *cagA* gene from biopsy DNA preparations, 1µl of DNA preparation was used in a reaction volume of 50µl. An identical reaction mix was used, but with 40 cycles of amplification.

2.7.3 Detection of the *vacA* gene.

vacA gene sequences were detected using the primer sets described by Atherton and co-workers (Atherton *et al*, 1995) as shown in Table I.

For bacterial chromosomal DNA preparations about 500ng of DNA was used in a volume of 1µl. The reaction mix was prepared as described above. The primer sets for m1, m2, s2 and universal s region types were used at 10pmol, and those for s1a and s1b types at 50 p mol.

The amplification protocol was a slightly modified version of that described previously (Atherton *et al*, 1995). 35 cycles of 95°C for 60s, 56°C for 60s and 72°C for 60s were used for bacterial chromosomal DNA samples. For biopsy DNA preparations, 1µl of DNA

preparation was used in a reaction volume of 50μl. The reaction mix was prepared as above, but using 40 cycles of amplification.

2.7.4 Tris-Acetate-EDTA (TAE) Agarose Gel Electropheresis.

PCR products were analysed by TAE gel electrophoresis and DNA visualised by ethidium bromide staining and UV transillumination.

15μl of the DNA sample was added to 5μl of DNA loading Buffer and the resulting sample was loaded on 2-3% agarose gels (Molecular Biology Grade; Sigma Chemical Company). The gels were run at 80V constant voltage for 60min. Bands were stained with ethidium bromide (25μg/ml) and visualised by UV transillumination.

2.8 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis.

2.8.1 SDS-PAGE.

SDS-PAGE was conducted using the Mighty Small II minigel apparatus (Hoefer Scientific) using discontinous gels as described by Laemmli (Laemmli, 1970).

Proteins were fractionated and separated using 7.5% acrylamide gels. Bacterial antigen preparations were mixed with 3x SDS-PAGE loading buffer and boiled for 5min before loading. Approximately 1-5μg of total protein was loaded per track, and gels run at 200V constant voltage for 45min, until the tracking dye run off the end of the gel.

2.8.2 Silver Staining.

Proteins were visualised by silver staining, using the Biorad Silver Stain Plus kit (Bio-rad), according to the manufacturers instructions.

Gels were fixed for 20min in Fixer Enhancer Solution, with gentle agitation on a rocking platform, then washed twice (10min each) with de-ionised water. The silver staining solution was added, and band development allowed until the desired staining intensity was achieved (7-10min). Staining was stopped by placing the gel in 5% (v/v) acetic acid solution for at least 15min.

2.9 Western Blotting.

2.9.1 Protein Transfer.

Proteins were electrophoretically transferred from the SDS-PAGE gels to Hybond-C super nitrocellulose membranes (Amersham International) using the Towbin buffer system (Towbin *et al*, 1979). Proteins were transferred using a Semi-Phor^{TM} semi dry blotter (Hoefer Scientific).

For transfer, Whatman filter paper (3mm) and Hybond-C super nitrocellulose membranes were cut to the same size as the polyacrylamide gel, and pre-soaked in transfer buffer. The transfer sandwich consisted of (from the anode) three pieces of Whatman filter paper, the nitrocellulose membrane, the gel and two more sheets of filter paper at the cathode.

Transfer was for 90min at 50mA, constant current. After transfer, membranes were blocked overnight at 4°C in 5% (w/v) skimmed milk powder in PBS.

2.9.2 Immunodetection.

2.9.2.1 Washing.

Blocked membranes were rinsed briefly with wash buffer, then washed for 15min on a rocking platform. The wash buffer was replaced with fresh buffer, and the membrane washed for a further 5min. This step was repeated with fresh buffer for a final 5min.

2.9.2.2 Primary antibody.

Washed membranes were incubated for 1h at room temperture on a rocking platform with the primary antibody, diluted appropriately. For testing human serum samples for the presence of antibodies to *H. pylori*, samples were diluted 1/100 in PBST, unless otherwise stated. For the separation of tracks, membranes were clamped in mini Deca-Probe units, and 500µl of diluted serum sample used per track.

Where a single serum sample was used against a number of different antigen preparations, the incubation was carried out in a sandwich box using a volume of at least 5ml.

For detection of the CagA protein using a specific mouse monoclonal (LDS56; Stephens *et al*, 1996), the antibody was diluted 1/10,000 in PBST unless otherwise stated. A volume of 10ml was used, in a sandwich box, and the membrane incubated for 1h at room temperature on a rocking platform.

2.9.2.3 Secondary antibody.

Membranes were washed as described previously and incubated for 1h at room temperture on a rocking platform.

Human primary antibodies were detected using a goat-anti-human IgG- peroxidase conjugate (Sigma) diluted 1/1000 in PBST, unless otherwise stated.

The mouse monoclonal antibody LDS56 was detected using a goat-anti-mouse IgG-peroxidase conjugate (Sigma) diluted 1/1000 in PBST unless otherwise stated.

2.9.2.4 Substrate Development.

Membranes were washed in PBST as before, but with 4x 5min washes. Band development was achieved using 3,3' diaminobenzidine (DAB) substrate solution (Sigma).

A 10mg tablet of 3,3'- diaminobenzidine tetrahydrochloride was dissolved in 15ml of PBS pH 7.4, and immediately before use, 12.5µl of hydrogen peroxide solution (H_2O_2 200 volume 60% w/v; Fisher Scientific) added. The substrate mix was then poured onto the filter in a sandwich box, and colour development allowed for up to 1h. The reaction was stopped by rinsing the filter in distilled water.

2.9.2.5 ECL™ detection.

As an alternative to DAB stain detection, the ECL detection system (Amersham) was used.

Membranes were washed as described previously and drained well by touching the edge of the membrane on tissue paper to remove excess wash buffer, but taking care not to let the membrane dry out. The ECL reagents were mixed using an appropriate volume for the size of the membrane (using 0.125ml/cm^2 membrane) according to the manufacturers instructions. The membranes were exposed to the detection reagent for exactly 1min and the excess reagent drained off. The membranes were wrapped in cling film and exposed to Hyperfilm-ECL (Amersham) for 10-50s and films developed in an Agfa 60 processing machine (Agfa Gevaert).

2.9.3 A commercial Western Blot kit for antibodies to *H. pylori* in human serum.

A commercial western blot kit (Helicoblot 2.0, Genelabs Diagnostics) was used for the detection of anti-*H.pylori* antibodies, in particular the anti-CagA antibody and the anti-cytotoxin antibody.

2.9.3.1 Preparation of Reagents.

Reagents were prepared as directed by the manufacturer. The wash buffer concentrate was diluted to 1 in 20 with Reagent water. The working blotting buffer was prepared by diluting the stock buffer concentrate 1 in 10 with Reagant water and then 1g of blotting powder added to every 20ml of stock buffer.

2.9.3.2.Assay.

The assay was carried out according to the maufacturers instructions.

The nitrocellulose strips, containing *H. pylori* lysate and a serum addition control band, were placed in the numbered wells. Strips for the positive and negative control serum were included in each of the assays. 2ml of the diluted wash buffer was added to each well and incubated for 5min at room temperature on a rocking platform. The buffer was removed and 2ml of working buffer and 20μl of patient serum were added to the wells. The samples were incubated for 1h on a rocking platform at room temperature, the mixture aspirated and a series of three washes with the diluted wash buffer was carried out. The working conjugate solution was prepared by diluting the conjugate 1:1000 into working blotting buffer. 2ml was added to each well and incubated for 1h, as before. The strips were washed as before, 2ml of working substrate solution added and incubated for 15min on the rocking platform. The substrate was aspirated and the strips rinsed with reagent water to stop further reaction. The resulting strips were dried and mounted.

2.10 Protein Determination.

The protein concentration of the bacterial antigen preparations was estimated by protein assay, using Coomassie Plus protein assay reagent (Pierce and Warriner) according to the manufacturers instructions.

Samples were diluted 1/100 in distilled water for a volume of 1ml. An equal volume of assay reagent was added and the tubes mixed by inversion. The absorbance was measured at 595nm using a UV spectrophotometer. Protein concentration was determined from a bovine serum albumin (BSA) standard curve of 0, 1, 5, 10, 15, 20 and 25μg/ml concentrations.

2.11 Enzyme-Linked Immunosorbent Assay.

A commercial ELISA kit for the semi-quantative determination of specific human IgG antibodies against the CagA protein of *H. pylori* was obtained form Shield Diagnostics, UK.

The ELISA kit 'Helicobacter pylori p120 (CAGA) ELISA (Viva Diagnostics) used a recombinant fragment of the CagA protein.

The assay was carried out according to the manufacturers instructions. The kit was validated for our population and for its use with whole blood samples, by Western blotting. The materials provided in the kit are detailed in Appendix III.

2.11.1 Assay Proceedure.

The sample diluent was diluted 1:10 with distilled water and used to dilute each blood sample 1:100. 100µl of each diluted sample, negative control sample and positive control sample were added to the appropriate microwells and the plate incubated for 1h at 37°C in a humid chamber. The wash buffer concentrate was diluted 1:20 with distilled water. After incubation, the microwell contents were aspirated and the wells washed 3 times. The wells were washed by filling the wells with diluted wash buffer, tipping out and tapping the plate dry on absorbent paper. The conjugate concentrate was diluted 1:2000 in sample diluent, 100µl added to each microwell and incubated at 37°C in a humid chamber for 1h. The microwells were decanted and washed as before. The substrate was diluted 1:2 with substrate diluent buffer, 100µl added to the wells and the plate incubated at room temperature for a further 30min. The reaction was stopped by the addition of 100µl of stop solution. The plates were read at 450nm using a Wellscan microplate reader (Denley Instruments Ltd) and the results converted to unit value according to the manufacturers instructions.

Chapter 3

in vivo typing of *cagA* status and *vacA* genotypes and their association with disease.

ABSTRACT.

Background- The relationship of *H. pylori* genotypes to gastrointestinal disease has relied on cultured isolates. This assumes that cultured strains are representative of *in vivo* strains.

Objective- To detect and type the *cagA* status and the *vacA* genotypes directly from biopsy DNA without the need for culture, and to further define the relationship between *H. pylori* genotypes and gastroduodenal pathology.

Methods- 61 patients undergoing routine endoscopy for dyspepsia had antral biopsies taken for biopsy DNA preparation and culture. In 52 of the patients, additional biopsies were taken from 4 gastric sites and 1 duodenal site. All recruited patients were *H. pylori* positive on rapid urease test for *Campylobacter* like organisms (CLO test) and/ or histology. The *cagA* status and the *vacA* s and m types were detected directly from biopsy DNA and cultured isolate DNA, by PCR amplification.

Results – The *cagA* and *vacA* alleles were successfully typed directly from biopsy DNA. *H. pylori* isolates were cultured from 30/61 patients in whom infection was detected by

PCR. There were discrepancies between the isolate and biopsy type in 7 of these 35 patient (20%). In 47/52 patients, strains were typeable from all four gastric sites and in 51/52 the same strain predominated through out. There was a correlation between *cagA* positivity and *vacA* s1 (47/49). Five of the 6 cancer strains were *cagA* positive/ *vacA* s1 type. There was no significant association between *cagA* or *vacA* s1 type and the presence of active peptic ulcer disease. Only 5/61 strains were of the type *vacA* s2/ m2 and 4 were in the non-ulcer dyspeptic (NUD) group.

Conclusion - *cagA* status and the *vacA* s and m alleles were successfully typed directly from gastric and duodenal biopsy DNA. Discrepancies between isolate and biopsy strain types stress the need for caution when interpreting *in vitro* strain types and suggest that direct PCR of biopsy DNA is the preferred typing technique. The *cagA* status and the s1 *vacA* allele are unreliable as single markers in determining the risk of developing peptic ulcer disease.

3.1 Introduction.

Two bacterial virulence determinants of *H. pylori* have been identified as potential markers of strain pathogenicity; the vacuolating cytotoxin gene (*vacA*) and the cytotoxin associated gene (*cagA*). The identification of *cagA* and *vacA* may be important in determining which infected individuals are at risk of developing serious gastrointestinal pathology.

The *vacA* gene encodes an 87-kDa protein, the vacuolating cytotoxin. The *vacA* gene is present in all strains of *H. pylori,* but active toxin expressed in approximately 50%. Several families of *vacA* have been identified and specific genotypes correlate *in vitro* with cytotoxin activity and the presence of gastroduodenal pathology (Atherton *et al*, 1995).

The *cagA* gene is present in 30-90% of *H. pylori* strains (Perez-Perez *et al*, 1997), and encodes a high molecular weight protein, the function of which is unknown (Covacci *et al*, 1993; Tummuru *et al*, 1993). The presence of *cagA* is closely associated with *in vitro* cytotoxin activity but insertional mutagenesis of *cagA* fails to disrupt toxin expression (Tummuru *et al*, 1994).

3.1.1 Typing of *vacA* genotypes and the *cagA* gene.

Previous work identifying the *vacA* genotypes and *cagA* gene as potential virulence determinants has been performed on bacterial isolates cultured from gastric biopsy samples using PCR amplification (Atherton *et al*, 1995). This introduces a potential bias towards readily cultured *H. pylori* strains and assumes that cultured (*in vitro*) strains are representative of *in vivo* strains. Only one study to date, involving 31 patients, has typed the *cagA* gene directly from gastric biopsy tissue by PCR amplification (Lage *et al*, 1995), only 11 of who had pathology (DU). There is no data on the typing of *vacA* subtypes from biopsy tissue.

3.1.2 Site specificity of strain types.

Bacteria have been typed predominantly from one gastric site, the antrum. This assumes that the same strain colonises all areas of the stomach and the duodenum. It is known that patients can be infected with multiple strains (Fujimoto *et al*, 1994), but there is no data looking specifically at whether certain strains can selectively colonise specific sites of the upper gastrointestinal mucosa. It has not been established if the antral strain type is representative of strain type throughout the stomach.

3.1.3 *cagA* and *vacA* in different study populations.

The initial study identifying the families of *vacA* and their association with *cagA* and gastroduodenal pathology was based in the USA (Atherton *et al*, 1995). The significance of these potential bacterial virulence determinants with gastroduodenal pathology in different geographical populations needs to be determined.

3.1.4 Aims to be tested:

1. to establish if it is possible to directly type infecting *H. pylori* strains for *cagA* and *vacA* genotypes from biopsy DNA, without the need for culture.
2. to compare biopsy strain types (*in vivo)* with isolate strain types (*in vitro*).
3. to assess if the strain type differs according to gastric and duodenal site.
4. look at the association of *vacA* genotypes and *cagA* with PUD and gastric cancer in a dyspeptic UK population.

3.2 Methods and Materials.

Patient selection and clinical assessment (section 2.2), histological assessment (section 2.3), bacterial culture and antigen preparation (section 2.5), DNA preparation (section 2.6) and PCR typing (section 2.7) are detailed in Chapter 2.

3.2.1 Statistics.

The results were analysed using the Chi squared test with Yates continuity correction and Fisher Exact Test.

3.3 Results.

3.3.1 Subject details.

A total of 61 symptomatic patients were studied: 38 male and 23 female. All except one of the patients were Caucasian. Patients were in the age range of 20-88. Twenty-four patients had NUD, 21 active DU, 10 active GU and 6 gastric carcinoma. Strain type in relation to site was looked at in a series of 52 of these 61 patients: 22 NUD, 16 DU, 9 GU and 5 gastric carcinoma (M: F ratio 32:20, age 20-87).

3.3.2 Detection of *H. pylori* infection.

Table 3.1 summarises the results of detection of infection with *H. pylori* by PCR, histology, rapid urease test (CLO) and culture in 61 subjects. Infection was detected by PCR in all 61 patients who were *H. pylori* positive by histology and/or rapid urease test. Isolates were cultured in 30/61 patients (49%) of patients. Biopsy specimens from 11 symptomatic subjects, who were *H. pylori* negative on histology, rapid urease test and culture, were tested for the presence of *H. pylori* sequences by PCR. These patients were all negative on PCR

Table 3.1-Comparison of *H. pylori* infection detection by PCR, CLO, histology and culture in 61 patients

Patient group	PCR	CLO test	Histology	Culture
NUD (n=24) positive	24	21	21	11
DU (n=21) positive	21	21	20	14
GU (n=10) positive	10	7	10	3
Ca (n=6) positive	6	5	6	2
Total (n=61)	61	54	57	30

3.3.2.1 PCR detection

H. pylori cagA and *vacA* s and m sequences were successfully detected by PCR in biopsy DNA and isolate DNA. A representative sample of *vacA* and *cagA* amplicons were directly sequenced at the Protein and Nucleic acid laboratory (PNACL), Leicester University to ensure that the primers used, correctly amplified the target sequences. For each set of PCR reactions, a positive (*H. pylori* chromosomal DNA) and a negative control (no DNA) were included, to check for suitable reaction conditions and that accidental contamination of the samples did not occur. In 10 patients, sterile water was flushed through the endoscopy channel (post cleaning) and then underwent PCR amplification; in all 10 patients the water was negative for the *vacA* and *cagA* sequences.

Figure 3.1 A 2% TAE agarose gel showing the *vacA* gene PCR products and 100bp molecular ruler; *vacA* m1 (290bp), m2 (352bp) and *vacA* s1a (190bp), s1b (187bp) and s2 (199bp).

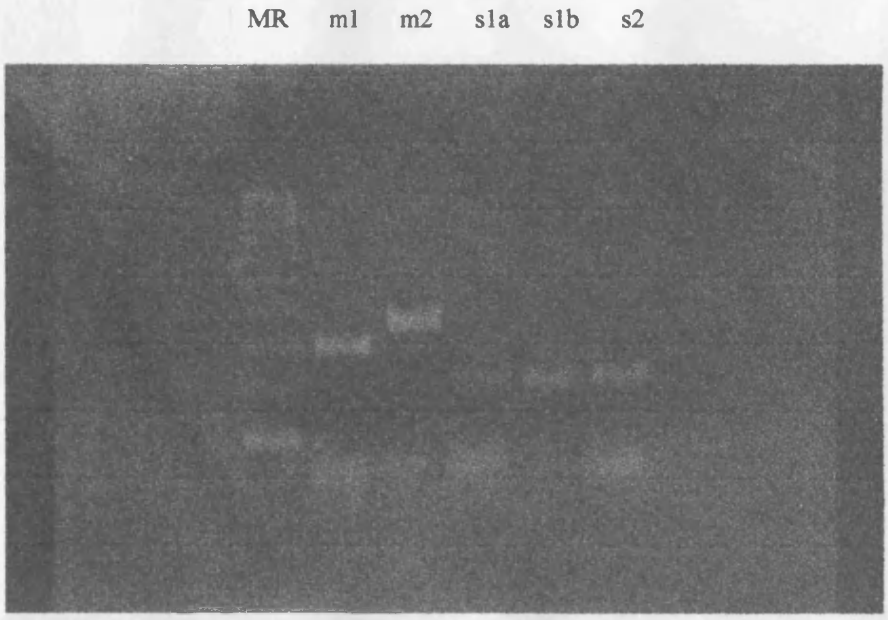


Figure 3.2 2% TAE agarose gels of *cagA* gene PCR products. Each gel has 9 patient biopsy DNA samples and a molecular ruler 100bp-1000bp.

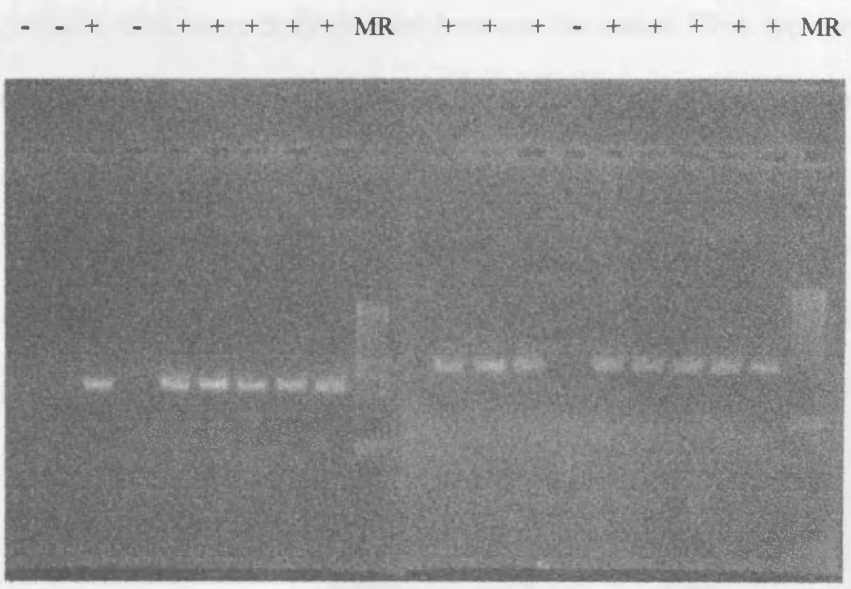
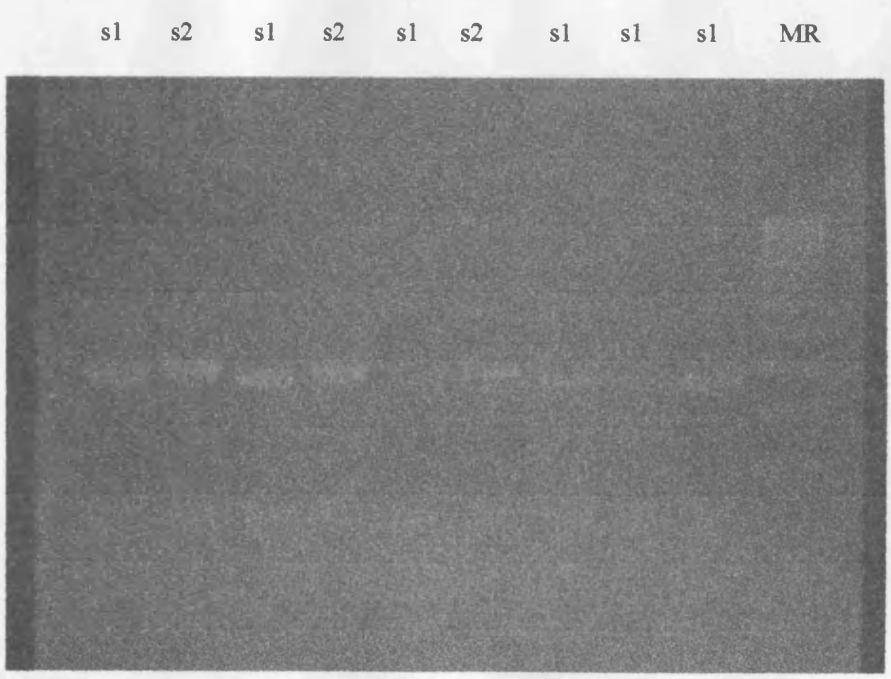


Figure 3.3 A 3% TAE agarose gel of PCR products from 9 samples with the *vacA* s region universal primers (s1 259bp: s2 286bp)



3.3.3 Discrepancies between culture and biopsy type.

Table 3.2 shows all the isolate types and compares them with the biopsy DNA types from the same patients. In 7 of the 31 patients (22.6%) in whom isolates were successfully cultured, there were discrepancies between the isolate DNA type and the biopsy DNA type. In 4 of these 7 patients, the isolates were of a different type to that detected by PCR on the biopsy sample. Two of these 4 patients differed between the isolate and the biopsy DNA type both in terms of the *cagA* status and the *vacA* type. In the two other patients, one differed in *cagA* status and the other differed in the *vacA* s1 type. The remaining 3 patients in whom there were discrepancies, the isolate DNA or the biopsy DNA did not type for the *vacA* m type.

Table 3.2 – Comparison of biopsy and isolate *cagA* status and *vacA* s and m types in 31 strains

Patient ID	Strain code	Strain type	Biopsy type
1	JS127	cagA+/s1	cagA+/s1
2	JS128	cagA-/s1m1	cagA-/s1m1
3	JS129	cagA+/s1m2	cagA+/s1m2
4	**JS131**	**cagA-/s1m2**	**cagA+/s1m2**
5	JS132	cagA-/s2m2	cagA-/s2m2
6	JS135	cagA+/s1m1	cagA+/s1m1
7	JS138	cagA+/s1m1	cagA+/s1m1
8	JS144	cagA+/s1m2	cagA+/s1m2
9	JS146	cagA+/s1m2	cagA+/s1m2
10	JS149	cagA+/s1m1	cagA+/s1m2
11	JS154	cagA+/s1m1	cagA+/s1m2
23	JS130	cagA+/s1m2	cagA+/s1m2
24	JS134	cagA+/s1	cagA+/s1
25	**JS136**	**cagA-/s1m1**	**cagA+/s1 m1,m2**
26	JS137	cagA+/s1m2	cagA+s1/m2
27	JS142	cagA-/s1m2	cagA-/s1m2
28	**JS143**	**cagA+/s1**	**cagA+/s1/m1**
29	JS145	cagA-/s1m2	cagA-/s1m2
30	**JS147**	**cagA+/s1a,m2**	**cagA+/s1a+b,m2**
31	JS148	cagA+/s1m2	cagA+/s1m2
32	JS150	cagA+/s1m2	cagA+/s1m2
33	**JS151**	**cagA+/s1m2**	**cagA+/s1**
34	JS153	cagA-/s1m2	cagA-/s1m2
39	JS140	cagA+/s1m1	cagA+/s1m1
40	JS141	cagA+/s1m2	cagA+/s1m2
41	**JS152**	**cagA+/s1**	**cagA+/s1m2**
48	JS133	cagA+/s1m2	cagA+/s1m2
49	JS139	cagA+/s1m1	cagA+/s1m1
54	**JS156**	**cagA-/s1m1**	**cagA+/s1+s2 m1+m2**
59	JS155	cagA+/s1m1	cagA+/s1m1
61	JS157	cagA-/s1m2	cagA-/s1m2

Red type represents discrepancies between isolate and biopsy type

3.3.4 Site and strain type.

3.3.4.1 Strain type according to site.

In 52 patients, strains from four gastric sites (antrum, antral-body junction, body and fundus) and the duodenum were compared. Table 3.3 shows the distribution of typeable strains between the different sites and is classified according to pathology. *H. pylori* genotypes were identified from all four gastric sites in 47/52 patients (90%). Fundal samples from two patients, and a body sample from one patient, failed to type for *H. pylori* sequences with PCR. In the remaining two patients, fundal samples were not obtained at endoscopy. Amplicons were obtained in only 16 of the 52 duodenal samples examined by PCR: only four of those were from patients with DU.

H. pylori sequences were detected at the antrum in all 52 patients. In 51 (98%) of the patients, the genotype identified at the antrum correlated with the genotype throughout the stomach. In one patient, *vacA* s1m1 type was identified throughout the stomach and in addition ***vacA*** s1m2 was also identified from the antral sample.

Table 3.3 Distribution of successfully typed amplicons between the four different gastric sites and the duodenal site, and classification according to gastroduodenal pathology

SITE	NUD	DU	GU	Ca
4 gastric +ve	19	15	9	4
Duodenal +ve	9	4	2	1
fundal/body -ve	2	0	0	1
no fundal sample	1	1	0	0

3.3.4.2 Typing inconsistencies according to site.

Discrepancies in typing according to the sample site in 15 patients are detailed in table 3.4. In 8 of the patients, detection of *vacA* s type was inconsistent between sites and in two of these patients no *vacA* m type was detectable at any of the five sites. Three patients had discrepancies in both the *vacA* s and m types detectable at the different sites. Detection of the *cagA* amplicon differed between sites in 4 of the 15 patients.

Table 3.4 – Discrepancies in typing of *cagA* and *vacA* s and m types between biopsy site within 15 patients.

The discrepancies are shown in red type

SITE		cagA	s1	s2	m1	m2
ID13	1	–	–	–	–	–
	2	+	+	–	–	+
	3	+	–	–	–	+
	4	+	–	–	–	+
	5	+	–	–	–	+
ID50	1	–	–	–	-	–
	2	+	+	–	+	–
	3	+	–	–	+	–
	4	+	+	–	+	–
	5	–	–	–	–	–
ID51	1	+	–	–	–	–
	2	+	+	–	–	–
	3	+	+	–	–	–
	4	+	+	–	–	–
	5	+	+	–	–	–
ID17	1	+	–	–	–	–
	2	+	+	–	+	–
	3	+	+	–	+	–
	4	+	+	–	+	–
	5	+	+	–	+	–
ID36	1	+	–	–	–	–
	2	+	+	–	–	–
	3	+	+	–	+	–
	4	+	+	–	+	–
	5	+	+	–	+	–
ID18	1	–	–	–	–	–
	2	–	–	+	–	+
	3	–	–	+	–	+
	4	+	–	+	–	+
	5	–	–	+	–	+
ID4	1	–	+	–	–	+
	2	+	+	–	–	+
	3	+	+	–	–	+
	4	+	+	–	–	+
	5	+	+	–	–	+
ID5	1	–	–	+	–	+
	2	–	–	+	–	+
	3	–	–	–	–	+
	4	–	–	+	–	+
	5	–	–	+	–	+

site		cagA	s1	s2	m1	m2
ID48	1	–	–	–	–	–
	2	+	..	–	–	+
	3	+	+	–	–	+
	4	+	+	–	–	+
	5	+	..	–	–	..
ID24	1	–	–	–	–	–
	2	+	+	–	..	..
	3	+	+	–	..	..
	4	+	.	–	..	..
	5	+	+	–	..	..
ID49	1	–	–	–	–	–
	2	+	+	–	+	–
	3	+	+	–	+	–
	4	+	+	–	+	–
	5	+	..	–	+	–
ID20	1	+	..	–	–	+
	2	+	+	–	–	+
	3	+	+	–	–	+
	4	+	+	–	–	+
	5	+	..	–	–	+
ID31	1	+	+	–	–	+
	2	+	+	–	–	+
	3	+	+	–	–	+
	4	+	+	–	–	+
	5	+	..	–	–	+
ID38	1	–	–	–	–	–
	2	+	..	–	–	+
	3	+	+	–	–	+
	4	..	.	–	–	+
	5	+	..	–	–	+
ID34	1	–	–	–	–	–
	2	..	+	–	–	+
	3	..	+	–	–	+
	4	+	+	–	–	+
	5	+	+	–	–	+

3.3.5 Strain type and disease.

Table 3.5.1 shows the distribution of *cagA* positive and negative strains in the different disease groups. The majority of strains were *cagA* positive (48/61, 79%). Five of the 6 gastric cancers were *cagA* positive. Sixteen of 21 (76%) duodenal ulcers, 7/10 (70%) gastric ulcers and 20/24 (83%) non-ulcer dyspeptics were *cagA* positive. The *cagA* gene was not significantly associated with ulcer strains compared to non-ulcer strains ($p=0.662$) (Table 3.5.2).

Table 3.5.1 – Distribution of *cagA* positive and negative strains according to gastroduodenal pathology in 61 patients.

DISEASE TYPE	*cagA+*	*cagA-*	Total
NUD	20	4	24
DU	16	5	21
GU	7	3	10
Ca	5	1	6
Total (n=61)	48	13	61

Table 3.5.2 – Distribution of *cagA* positive and negative strains in patients with and without active peptic ulcer disease

DISEASE TYPE	*cagA+*	*cagA-*
PUD	23	8
NUD	20	4

Strain type in association with disease type is shown in Table 3.6. Thirty of the 31(97%) PUD strains were type *vacA* s1, compared with 20/24 NUD strains (83%), (p= 0.107). Thirty-three of 61 strains (54%) were *vacA* m2 types. In the duodenal ulcer group, 12/21 types (57%) were m2 and 2/12 contained both m1 and m2 types. The majority of the NUD strains were *vacA* m2 (15/24, 63%); there was no association between *vacA* m type and gastroduodenal ulceration. Six of the 61 strains were non-typeable for the *vacA* m1 and m2 regions. Five of the 61 patients were infected with *vacA* s2/m2 strains. Of these, 4 were from the NUD patient group. Ninety eight percent (47/49) of the *cagA* positive strains were *vacA* s1 type. Five out of 6 gastric cancer strains were *cagA* positive/*vacA* s1 type. Seventy four percent (23/31) of the ulcer strains were *cagA* positive/*vacA* s1 type compared with 79% (19/24) of the non-ulcer patients.

Table 3.6 – Distribution of *vacA* s and m types in *cagA* positive and negative strains, according to gastroduodenal pathology.

STRAIN TYPE		NUD	DU	GU	Ca
cagA+	s1m1	6	3	5	2
	s1m2	11	8	2	2
	s1 only	2	3	0	1
	s2m1	0	0	0	0
	s2m2	1	0	0	0
cagA-	s1m1	1	1	1	1
	s1m2	0	3	2	0
	s1 only	0	0	0	0
	s2m1	0	0	0	0
	s2 m2	3	1	0	0
Mixed s and m types:					
cagA+: s1/s2 & m1/m2		0	1	0	0
s1, m1/m2		0	1	0	0
Total (n=61)		24	21	10	6

3.4 DISCUSSION.

This work looked at 61 patient samples from a homogenous dyspeptic Leicester population and attempted to type the *cagA* status and the *vacA* genotypes directly from biopsy DNA in order to assess if *in vitro* strains are representative of *in vivo* strains.

3.4.1 Typing of the *cagA* and *vacA* directly from biopsy DNA by PCR.

The PCR amplification technique and primers used in this study have been previously successfully used on bacterial isolates in a previous study based in Leicester (Stephens *et al*, 1998), but the technique was not attempted directly on biopsy DNA. The current work demonstrates that the *cagA* status and the *vacA* s and m regions can be successfully typed by PCR amplification directly from biopsy DNA, in *H. pylori* infected individuals.

Although previous studies have detected the urease gene directly from gastric biopsy samples using PCR (Bickley *et al* 1993; Lin *et al*, 1996) direct amplification of the *vacA* genotypes and the *cagA* gene from gastric biopsies has not been studied in detail. There have been no previous studies on the typing of *vacA* genotypes directly from biopsy samples. This is the first study to demonstrate that it is possible to type the *vacA* alleles directly from biopsy DNA using PCR amplification. All combinations of the *vacA* genotypes were identified with the exception of s2/m1. This is consistent with the studies that have identified *vacA* s and m types using cultured bacterial DNA (Atherton *et al*, 1995). Only one other group (Lage *et al*, 1995) have typed the *cagA* gene directly from gastric biopsies using PCR. Lage and colleagues analysed biopsy samples and isolates from 31 patients, compared to 61 patients studied in the current work, and were able to successfully type *cagA* directly from gastric biopsies. Typing from duodenal biopsies was

not studied. They demonstrated a correlation in all 31 patients between direct amplification of the *ureC* and *cagA* fragments from biopsies and from the corresponding isolate. These results differ from the findings of the current study, as discussed below.

A series of eleven *H. pylori* negative subjects, by histology, culture and rapid urease test, were included as a control group. The lack of detection of the *cagA* and *vacA* s and m PCR sequences in these patients demonstrates that cross reactivity of sequences from human DNA are unlikely to give false-positive PCR results for these primers.

3.4.2 Culture

Isolates were cultured from 49% (30/61) patients who were successfully typed for the *cagA* gene and the *vacA* genotypes. The low success rate of culture in this study, and in others (Lage and colleagues culture rate was 36.5%, [Lage *et al*, 1995]), is an important point that highlights the difficulties with relying on culture as a means of diagnosing *H. pylori* infection. A single experienced microbiologist cultured all the isolates in this study. Both selective and non-selective media were used to maximise the culture success rate (Piccolomini *et al*, 1997). Temperature and length of freeze time influence the culture yield and are important variables to consider in this study. Gastric mucosal biopsies for culture were stored temporarily in a fridge, and were frozen at – 20 °C, within 1-8 hours of being taken. The biopsies were thawed and cultured within 1-2 weeks; biopsies thawed within four weeks of freezing should have up to a 100% isolate success rate (Han *et al*, 1995). Culturing the organisms immediately after taking the biopsies would have increased the culture success rate, but it is rare to have this facility in a normal busy clinical laboratory.

Only one antral sample was taken from each patient for culture. *H. pylori* colonisation can be patchy in nature, so it is possible that, in a proportion of patients, the antral biopsies taken for culture were only scantily or not colonised by the bacteria. This is an argument for taking multiple biopsies from different sites in order to maximise the rate of culture success.

3.4.3 Comparison of isolate and biopsy strain types.

The majority of isolate strain types correlated with the biopsy strain types, but in 22.6% there was discrepancy, contrary to the findings of Lage and colleagues (Lage *et al*, 1995). The presence of multiple infections is the most likely explanation for this discrepancy and is well-documented (Fujimoto *et al*, 1994). The detection of multiple *vacA* types in the biopsy DNA in three of the patients suggests colonisation with multiple strains. The discrepancy in the *cagA* status between cultured isolate and biopsy samples again suggests infection with a mixed population of *cagA* positive and *cagA* negative *H. pylori* strains. A recent study demonstrated that four of eight patients infected with *H. pylori*, were infected with a mixture of *cagA* positive and *cagA* negative types (Van der Ende *et al*, 1998). The possibility of deletion of cagA-containing sequences *in vitro,* or of false *cagA* negative results (Owen *et al*, 1994), cannot be excluded as an explanation for the discrepancies in *cagA* status seen in this study.

3.4.4 Site, *vacA* and *cagA*.

Although multiple strains can colonise an individual, it has not been established whether certain strain types selectively colonise certain areas of the stomach. In this study, the results suggest that the same strain predominates throughout all areas of the gastric mucosa. Failure to detect additional *vacA* types throughout other areas of the stomach suggests that antral sampling is a suitable area to use in clinical practice for identifying infecting strain type. It is important to note that DNA typing does not directly correlate with established colonisation of the strains at these sites.

Caution is needed in interpreting these findings as potential contamination from site to site during biopsy may bias these results. In order to eliminate cross contamination between sites and to clarify accurately the relationship between strain type and gastric site, biopsy forceps would need to be changed in between sites. Inter-patient contamination is a possibility but unlikely. The recommended BSG endoscopic and forceps cleaning protocols were vigorously followed and in all 10 patients in whom water was flushed though the endoscopy channels the water was PCR negative for the *cagA* and *vacA* amplicons.

It is possible to type the *cagA* gene and the *vacA* genotypes from duodenal biopsy samples, but the detection of *H. pylori* in the duodenum bears no relationship to the presence of duodenal ulceration. Explanations for this lack of association include patchy colonisation of *H. pylori* in the duodenum and false negative PCR results (Owen *et al*, 1994).

Inconsistencies between sites was noted in 15 patients. A poor yield of DNA is the most likely explanation, especially if the biopsy sample was small. A low density of bacterial colonisation and thus small amounts of bacterial DNA, and differing primer efficiency for different reactions are other possible explanations.

3.4.5 *vacA, cagA* and disease

3.4.5.1 *cagA.*

A relationship between *cagA* positive strains and gastric carcinoma has been shown in numerous studies both serologically and from cultured isolates (Blaser *et al*, 1995; Parsonnet *et al*, 1997). The *cagA* gene has also been shown to be associated with gastroduodenal ulceration (Cover *et al*, 1995), and anti-CagA antibodies are associated with the gastric cancer precursors, intestinal metaplasia and gastric atrophy (Sozzi *et al*, 1998). However, the association of *cagA* with disease type varies with the geographical population studied, and is becoming less distinct as more data accumulates. In Japan and China, *cagA* strains are very common both in patients with and without PUD and gastric cancer, implying that *cagA* is not an important virulence factor or marker in these populations (Maeda *et al*, 1997; Pan *et al*, 1997; Shimoyama *et al*, 97). A study of CagA seropositivity in several different countries showed no association between the variation in CagA positivity rates and gastric carcinoma (Perez-Perez *et al*, 1997), although this differed from the findings of The Eurogast study group (Eurogast Study Group, 1993). More recently, a study of isolates from 19 patients with non-ulcer dyspepsia demonstrated that in 68% of cases, mixed colonies of *cagA* positive and *cagA* negative existed (Figura *et al*, 1998). In this study, a correlation was seen between gastric cancer and *cagA* (although the number of gastric cancer patients was small) but no significant difference was seen in the prevalence of *cagA* positive strains in patients with or without ulceration. Therefore, *cagA* it is not useful as a single marker of disease in this UK population.

3.4.5.2 *vacA*.

Isolates from patients with peptic ulcer disease in the USA have been shown to contain the s1 *vacA* alleles significantly more frequently than isolates from patients without peptic ulcer disease (Atherton *et al*, 1995). Although other studies have found similar findings to Atherton and colleagues (van Doorn *et al*, 1998; Warburton, 1998) the classifications of *vacA* subtypes and associations with PUD are more complex than shown in the original study.

Important differences have been demonstrated in different geographical populations. A study was carried out on a UK group of patients looking at the association of *vacA* types and peptic ulceration (Stephens *et al*, 1998) and demonstrated less convincing associations. It is interesting to note that the study was carried out in Leicester where 25% of the population are of Asian origin. Although the allele s1 correlated with PUD, there was also a high rate of s1 types in the non-ulcer dyspeptic (NUD) group (83% compared with 48% in Atherton's group). There were very few s2 types isolated, and the s2/m2 type was rare, only being present in the NUD group. Nine isolates were non-typeable for the mid region. Several recent studies have also shown found this heterogeneity in the *vacA* mid-region. A study in Germany found 18% of strains were untypeable for the mid-region, and hypothesised from their work that a third subfamily of sequences in the mid-region existed (Strobel *et al*, 1998). Genetic analysis of 119 isolates in Taiwan failed to type 15 strains for the *vacA* mid region with the primers from Atherton's initial study (Atherton *et al*, 1995), and identified further variability of the mid-region in these 15 isolates (Wang *et al*, 1998). van Doorn and colleagues demonstrated differences in the distribution of *vacA* s1a and s1b types in northern and eastern Europe compared with the Iberian peninsula (van Doorn *et al*, 1999) and also demonstrate further variation in both the s and m regions, identifying a s1c subtype (considered to be a recombinant of s1a and s1b) and m2a and m2b subtypes in East Asia isolates (van Doorn *et al*, 1998). Analysis of strain types in isolates from Japan and Taiwan has shown similar differences in *vacA* genotype sequences and the association

with gastroduodenal pathology. A study in Japan revealed that s1a/m1 strains were common in both non-ulcer dyspeptics and PUD, with no association between the different *vacA* types and *in vitro* cytotoxic activity (Ito *et al*, 1997). Further work in Japan showed similar results (Maeda *et al*, 1998). In Taiwan, sequence analysis showed heterogeneity of Taiwanese strains when compared to strains in other countries (Yang *et al*, 1997).

The current findings are in keeping with the recent data published and reveal a less convincing association between the *vacA* s and m types and disease type. All except one of the duodenal ulcer strains were *vacA* s1, but there was still a high proportion of the non ulcer strains which were also *vacA* s1 (83%), making *vacA* s1 unreliable as a marker of virulence in this study population. There was no difference in the distribution of *vacA* s1m1 and *vacA* s1m2 types between ulcer and non-ulcer patients. A previous study in Leicester demonstrated a low prevalence of *vacA* s2 types in the UK (Stephens *et al*, 1998) and similarly, only 5 *vacA* s2m2 strains were typed in this study. The *vacA* s2 type was more prevalent in the NUD patients (4/5) than in PUD patients, and although this failed to reach statistical significance, the rarity of the *vacA* s2 type in this population makes the statistical analysis unreliable (a potential type two error). However, this trend may still be an important one.

Six of the 61 strains were not typeable for the *vacA* m1 and m2 regions. This is consistent with the previous study in Leicester (Stephens *et al*, 1998), although it is important to note that ethnic minorities were included in the study by Stephens and colleagues, unlike the current work. Reasons for the non-typeable m-regions in this study include false negative molecular typing results, and the possibility of new-variants of the m-region have been shown in certain populations, as discussed above (Strobel *et al*, 1998; Wang *et al*, 1998).

vacA type s1m1 produces greater levels of cytotoxin activity *in vitro* than the *vacA* type s1m2 (Atherton *et al*, 1995) although the mechanisms for this cytotoxin phenotype

variation remain uncertain. Differences in *vacA* transcription levels between *H. pylori* strains has been implicated (Forsyth *et al*, 1998). More recently work by Letley and colleagues suggests that a 12 amino acid extension on the N-terminus of VacA from s2 strains alters toxin activity (Letley *et al*, 1999). Various groups have shown a relationship between cytotoxin production and the *cagA* gene (Covacci *et al*, 1993; Tummuru *et al*, 1993). We were unable to study the *in vitro* production of cytotoxin in these strains, but the results demonstrated a strong relationship between *cagA* positivity and the s1 *vacA* allele. There was no significant association between *cagA* positive/ *vacA* s1 type and PUD.

3.4.6 Criticisms and considerations for future studies.

Several points have been highlighted in the discussion and are detailed below.

The number of cancer patients in this study is low, and little can be concluded from this study as regards *H. pylori cagA* status and *vacA* genotypes and gastric cancer. Only trends can be noted. Further data is required in this population to confirm the trends.

As regards the biopsy sampling in this study, the number of biopsies taken for culture could have been increased to include both an antral and a body sample, and by processing the samples for culture immediately, the culture success rate may have been improved. This is certainly an option in the research setting, but would not be representative of using culture as a means of diagnosis in a clinical laboratory, and would potentially be misleading in terms of results. Changing of the biopsy forceps or cleaning and decontamination of forceps in-between biopsy sites may help to reduce the possible carry over of DNA from site to site in the stomach, and reduce the risk of inter-site contamination. The practicality of this, however, is questionable, as the length of the endoscopic procedure would be increased unacceptably to both the patient and the endoscopy staff.

The absence of *cagA* as detected by PCR could have been falsely negative. Ideally the negative results should have been repeated with empty sire primers based on sequence either side of *cag* PAI. This would give a product with only *cag*A negative strains.

negative PCR results of the 11 *H. pylori* negative patients, for *cagA* and *vacA* sequences with these primers, makes it unlikely that false positives result from cross reactivity with human DNA sequences. False positives from homologous sequences in other organisms is a consideration. In order to clarify that the PCR products were *cagA* and *vacA* s and m types, a sample of PCR products were formally sequenced. Southern hybridisation is an additional technique that could be employed.

In vitro cytotoxin expression of *H. pylori* isolates was not carried out in this study, but would have provided some interesting extra data. However, recent work (Pagliaccia *et al*, 1998) has shown that differences in receptor binding of different toxin types may cause misleading results, depending on which cell line is used for the crude *in vitro* cytotoxin assay.

3.4.7 Summary and significance of the findings

In conclusion, this work is the first to demonstrate that it is possible to determine the *vacA* s and m regions directly from gastric and duodenal biopsies, without the need for culture. The *cagA* status was also successfully determined directly from biopsy DNA. *In vitro* strains are not always representative of *in vivo* strains, especially if mixed infections are present, and caution is needed in interpreting strain type from cultured biopsies. These points emphasise that direct PCR amplification of biopsy DNA may in fact be the preferred PCR typing technique for defining the infecting strain of *H. pylori.* The detection of *H. pylori* in the duodenum using direct PCR bears no relationship to the presence of duodenal ulceration, but further studies are required to evaluate the significance of this finding.

Overall, there is a high *cagA* positivity rate amongst a dyspeptic homogenous population in Leicester. Similar high *cagA* positivity rates have been recorded elsewhere (Ito *et al*, 1997; Pan *et al*, 1997; Yang *et al*, 1997). In agreement with these studies, this data indicates that *cagA* status is an unreliable single marker of peptic ulcer disease, or of increased risk of gastric carcinoma. Additionally, the data shows that the *vacA* s1 allele is an unreliable marker of peptic ulcer disease, with the overwhelming majority of strains detected in this population being this type. Indeed, the *vacA* s2 allele may be a more reliable marker of patients unlikely to develop serious gastrointestinal disease, and the question of prevalence of the *vacA* s2 in the asymptomatic population remains undetermined.

Chapter 4

Inflammation, disease and *H. pylori cagA* and *vacA* genotypes.

ABSTRACT.

Background. The *cagA* status and specific *vacA* genotypes of *Helicobacter pylori* have been shown to correlate with bacterial density, extent of inflammation and peptic ulceration.

Aims. To assess the significance of *cagA* and *vacA* subtypes in relation to inflammation and density of bacterial colonisation *in vivo* within a dyspeptic UK population.

Methods. Dyspeptic patients who were *Helicobacter pylori* positive by rapid urease test (CLO) and/ or histology, had antral samples taken for histology and culture. Gastroduodenal pathology was noted. The grade of bacterial density and inflammation was assessed using the Sydney system. Bacterial DNA was extracted and the *vacA* alleles and the *cagA* gene typed using PCR.

Results. 120 patients were studied. The *cagA* gene and the *vacA* s1 allele were associated with PUD, although the overall prevalence of *cagA* positive and *vacA* s1 type strains was high in this population. Bacterial density did not correlate with the presence of peptic ulceration.

There was a significant association between *cagA* positive strains and increased inflammation and bacterial density. The *vacA* s1 type independently correlated with extensive chronic inflammation but there was no association with bacterial density. The *vacA* m type did not correlate with extent of inflammation or bacterial density. Eight strains were untypeable for the mid-region.

Discussion. The results of this study suggest that *cagA* is important in the pathogenesis of inflammation and peptic ulceration. These findings are in keeping with *cagA* as a marker for a '*cag* pathogenicity island' which encodes several genes involved in inflammation. The *vacA* s1 allele correlates with inflammation, independently of *cagA*, possibly via its enhanced ability to produce the vacuolating cytotoxin. Neither *cagA* nor the *vacA* s1 allele are reliable indicators of active ulceration. Heterogeneity of the *vacA* mid-region appears to exist in this population.

4.1 Introduction.

H. pylori is a major aetiological factor in the development of chronic active gastritis, PUD and gastric cancer. Infection with *H. pylori* invariably results in gastritis, but only a minority of individuals will go on to develop pathology. The severity of the gastric inflammation is an important determinant of disease outcome, and bacterial virulence determinants of the infecting strain may play a role in the pathophysiology of inflammation. The *vacA* genotypes and the *cagA* status of an infecting strain are potentially important bacterial agents involved in inflammation.

Specific *vacA* alleles correlate with *in vitro* cytotoxin production and the presence of peptic ulcer disease (Atherton *et al*, 1995). Cytotoxin positive strains compared to toxin negative strains are associated with an increase in antral polymorph infiltration (Cover *et al*, 1993). The *cagA* gene is recognised as a marker of enhanced virulence and it is thought to identify an island of genetic pathogenicity (Censini *et al*, 1996). The '*cag* pathogenicity island' encodes several genes involved in the induction of pro-inflammatory cytokines (Censini *et al*, 1996; Tummuru *et al*, 1995).

Several groups have noted a correlation between strains that possess *cagA* and the severity of gastric mucosal inflammation (Crabtree *et al*, 1995; Peek *et al*, 1995) but the patient groups have been small in number. More recently, work in the USA has demonstrated a relationship between specific *vacA* alleles and the extent of gastric inflammation (Atherton *et al*, 1997). At the time of this data analysis, a very recent study published the effect of *cagA* and the *vacA*

genotypes on the pattern of gastritis and the severity of inflammation (Warburton *et al*, 1998). One hundred and sixty one patients were included in the analysis, and no association between the bacterial factors and pattern of gastritis was demonstrated, but *cagA* positivity and *vacA* s1 type were found to correlate with severity of inflammation.

To further evaluate the relationship between *cagA* status, *vacA* genotypes and gastric inflammation, a group of symptomatic UK patients were studied.

4.1.1 Aims to be tested:

1. to evaluate the relationship between the *cagA* status, density of bacterial colonisation and severity of gastric inflammation
2. to establish the relationship between the *vacA* genotypes, density of bacterial colonisation and the extent of gastric inflammation
3. to look at the association of *cagA*, the *vacA* genotypes and gastroduodenal pathology in a symptomatic UK group of patients.

4.2 Methods and Materials.

Patient selection and clinical assessment (section 2.2), bacterial culture and antigen preparation (section 2.5), DNA preparation (section 2.6) and PCR typing (section 2.7) are detailed in Chapter 2.

4.2.1 Histology

The extent of bacterial density and grade of acute and chronic inflammation was assessed using the Sydney System (Dixon *et al*, 1996).

For the purposes of this study, we grouped together grades 0 and 1 to represent mild/ low density and inflammation and grades 2 and 3 to represent moderate/ severe. All the patients included in the study were *H. pylori* positive as determined by rapid urease test, culture and histology. Thus, grade '0' is a reflection of patchy distribution of bacteria and gastritis. This justifies the pooling of data to represent mild versus severe inflammation and low versus high density.

4.2.2 Statistics.

The results were analysed using the Chi squared test with Yates continuity correction and the Fisher Exact Test.

4.3 Results.

4.3.1 Patient groups.

The 52 patients discussed in Chapter 3, and 68 patients from a previous Leicester study (Stephens *et al*, 1998) were included this study. The 120 dyspeptic patients were mixed Caucasian and Asian origin, with and age range of 19-87 and a M:F ratio of 75:45. All the patients were *H. pylori* positive on rapid urease test and/or histology and were successfully typed for *cagA* and the *vacA* alleles. 74 were non-ulcer dyspeptics, 41 had peptic ulcer disease of which 16 were gastric ulcers and 25 duodenal ulcers, and 5 had gastric carcinomas.

4.3.2 *cagA* status and *vacA* genotypes.

The *vacA* s1 type is subdivided into s1a and s1b types (Atherton et al, 1995), but in this study the s1 type was looked at as a whole, because 90% of s1 stains were s1a in this population. Table 4.1 summarises the strain types in the study population. Ninety-one of 120 strains (76%) were *cagA* positive. The *vacA* genotypes of the strains were the following: 45 s1/m1, 52 s1/m2, 13 s2/m2 and 1 strain was non-typeable for the *vacA* s region and 8 strains non-typeable for the *vacA* m region. There were no strains of the type s2/m1. Eighty seven of 91 *cagA* positive strains were *vacA* s1 (95.6%) compared with 19/29 *cagA* negative strains (65.5%); the presence of *cagA* was significantly associated with *vacA* s1 type ($p<0.05$).

4.3.3 Strain types and pathology.

Table 4.1 shows the distribution of strain types amongst the different disease groups. The prevalence of *vacA* s1 type in NUD patients was 82.4%, compared with 97.6% of the PUD patients ($p=0.013$). There was a non-significant trend between *cagA* positive strains and PUD (83%) compared with NUD patients (70%), ($p=0.10$).

In patients with PUD, 34/41 strains (83%) were *cagA* positive and *vacA* s1 type. Within this group 11/16 GU strains were of the type *vacA* s1/m1 compared to 11/25 DU strains.

Forty eight of the 74 NUD strains were *cagA* positive/ *vacA* s1 (66.7%). 25 strains were s1/m1 compared to 32 type s1/m2. 13 strains were type s2/m2, only one of which was a strain from a patient with PUD. All 5 gastric cancer patients were *cagA* positive and *vacA* s1.

Table 4.1 – The distribution of *cagA* positive and negative strains and *vacA* s and m types according to disease type.

	All strains		NUD		PUD		Ca
vacA	*cagA* +	*cagA*-	*cagA*+	*cagA*-	*cagA*+	*cagA*-	*cagA*+
s1/m1	39	6	21	4	16	2	2
s1/m2	40	12	23	9	15	3	2
s2/m2	3	10	3	9	0	1	0
s1 only	8	1	4	0	3	1	1
m2only	1	0	1	0	0	0	0
Total	91	29	52	22	34	7	5

4.3.4 Peptic ulceration and bacterial density

The density of bacterial colonisation in the antrum was assessed in patients with and without ulceration (Table 4.2). Bacterial density was low (grades 0/1) in 33/74 of NUD strains and 20/41 of ulcer strains. There was no correlation between the density of bacterial colonisation and the presence of peptic ulceration ($p=0.8$). When data for DU and GU patients were analysed separately, the lack of association persisted.

Table 4.2 – Grade of bacterial density according to the presence or absence of peptic ulceration.

	Grade of bacterial density	
	0-1	2-3
NUD strains	**33**	**41**
DU strains	12	13
GU strains	8	8
PUD strains	**20**	**21**

4.3.5 *cagA* status, bacterial density and inflammation.

Bacterial density of colonisation and extent of inflammation were examined with reference to the *cagA* status of infecting strain (Table 4.3). In patients infected with *cagA* positive strains there was a denser colonisation of *H. pylori* in the antrum ($p=0.001$) and a significant increase in the inflammatory response when compared to patients infected with *cagA*- strains (chronic $p=0.003$, acute $p=0.011$).

The distribution of *cagA* positive and negative strains in relation to inflammation and bacterial density in patients with PUD is shown in Table 4.4. Twenty-four of the 34 *cagA* positive strains were associated with severe acute inflammation compared to 1/7 of the *cagA* negative strains ($p=0.009$). A similar statistically significant correlation was seen between *cagA* positive strains and bacterial density (21/34 *cagA* positive strains versus 0/7 *cagA* negative strains were associated with a dense colonisation, $p=0.003$). There was no association between chronic inflammation and *cagA*.

Table 4.3 –Analysis of *cagA* positive and negative, bacterial density and inflammation in all strains

cagA status	All Strains					
	Bacterial Density		Acute Inflammation		Chronic Inflammation	
	0-1	2-3	0-1	2-3	0-1	2-3
cagA + strains	34	57	42	49	15	76
cagA - strains	21	8	21	8	13	16

Table 4.4- Analysis of *cagA* positive and negative strains, bacterial density and inflammation in patients with peptic ulceration

cagA status	PUD					
	Bacterial Density		Acute Inflammation		Chronic Inflammation	
	0-1	2-3	0-1	2-3	0-1	2-3
cagA + strains	13	21	10	24	4	30
cagA - strains	7	0	6	1	2	5

The relation of *cagA* with inflammation and bacterial density was examined in NUD patients (table 4.5). In the NUD strains, there was a significant association between *cagA* positive strains, bacterial density (33/52 *cagA* positive versus 8/22 *cagA* negative strains were grades 2-3, $p<0.03$)) and chronic inflammation (42/52 *cagA* positive versus 11/22 *cagA* negative strains were grades 2-3, $p<0.009$).

Table 4.5 – Analysis of *cagA* positive and negative strains, inflammation and bacterial density in patients with non-ulcer dyspepsia

	NUD					
cagA	Bacterial Density		Acute Inflammation		Chronic Inflammation	
Status	0-1	2-3	0-1	2-3	0-1	2-3
cagA + strains	19	33	29	23	10	42
cagA - strains	14	8	15	7	11	11

4.3.6 *vacA* type, bacterial density and inflammation.

Table 4.6 summarises the *vacA* s and m types in terms of bacterial density and inflammation.

Table 4.6- Association of *vacA* s and m types with inflammation and density.

	All strains					
	Bacterial Density		Acute Inflammation		Chronic Inflammation	
	0-1	2-3	0-1	2-3	0-1	2-3
vacA m1	20	24	21	23	12	32
vacA m2	31	35	36	30	15	51
vacA s1	47	59	53	53	20	74
vacA s2	8	5	10	3	8	5

4.3.6.1 *vacA* s1

Type s1 strains were associated with a marked chronic inflammatory infiltrate (grades 2-3). There was no correlation of *vacA* s1 type and acute inflammation or density of bacterial colonisation. As *vacA* s1 and *cagA* are closely associated, it is important to analyse *vacA* s1 independently of *cagA*. Analysis of *vacA* type s1 in relation to *cagA* positive and *cagA* negative strains, showed that the association with severe chronic inflammation persisted ($p=0.035$), demonstrating that the association was independent of *cagA* (Table 4.7). A similar trend was demonstrated within the PUD strains, as shown in Table 4.8, but did not reach statistical significance ($p=0.588$).

Table 4.7 Association of *vacA* s1 with bacterial density and inflammation overall and independently of *cagA*.

All Strains						
vacA s1	Bacterial Density		Acute Inflammation		Chronic Inflammation	
	0-1	2-3	0-1	2-3	0-1	2-3
vacA s1	47	59	53	53	20	74
s1/*cagA*+	32	55	39	48	13	74
s1/*cagA*-	15	4	14	5	7	12

Table 4.8 – *vacA* s1 independent of *cagA* and chronic inflammation in patients with peptic ulcer disease

PUD strains		
vacA s1	Chronic Inflammation	
	0-1	2-3
vacA s1	5	35
s1/*cagA*+	4	29
s1/*cagA*-	1	5

4.3.6.2 *vacA* s2

The number of *vacA* s2 strains in this study population was very small (13/120, 10.8%). Thus, we were unable to reliably compare and contrast the relationship of the s2 genotype, inflammation and bacterial density with that of the s1 genotype.

4.3.6.3 *vacA* m types

There was no association between type m1 or type m2 and grade of bacterial density or inflammation independently of *cagA* (data in Table 4.6). Types m1 and m2 were further analysed (Table 4.9) and compared in patients with *cagA* positive strains and no significant difference was demonstrated between the mid- region sequences and the extent of inflammation or density of bacterial colonisation.

Bacterial density and inflammation were examined with reference to the *vacA* subtypes (table 4.10). Comparison of the type v*acA* s1m1 with *vacA* s1m2 showed no association between the two types in relation to inflammation or bacterial density. The number of s2 strains was very small in this study, and thus *vacA* s1m1 could not be reliably compared to *vacA* s2m2 in terms of inflammation or density, or independently of *cagA*. It is of interest however, that only one of the *cagA* positive s2m2 strains had high grade inflammation.

Table 4.9 Association of *vacA* m types with grades of inflammation and bacterial density in *cagA* positive strains.

All Strains						
m type	Bacterial Density		Acute Inflammation		Chronic Inflammation	
	0-1	2-3	0-1	2-3	0-1	2-3
m1/*cagA* +	16	23	18	21	10	29
m2/*cagA* +	15	29	20	24	5	39

4.10 – Association of the *vacA* genotypes with inflammation and density.

All strains						
vacA	Bacterial Density		Acute Inflammation		Chronic Inflammation	
genotypes	0-1	2-3	0-1	2-3	0-1	2-3
vacA s1m1	20	24	21	23	12	32
vacA s1m2	23	29	26	26	7	45
vacA s2m2	8	5	10	3	8	5
cagA+ s1m1	16	22	17	21	10	28
cagA+ s2m2	2	1	3	0	2	1

4.4 DISCUSSION.

This study involves a large number of dyspeptic patients from a UK population, and investigates the relationship between *vacA* genotypes and the *cagA* status with severity of inflammation *in vitro*. The concomitant use of non-steroidal anti-inflammatory drugs (NSAID drugs) was an important exclusion criteria.

4.4.1 Strain types.

Overall, there was a high *cagA* positivity rate in the study population (76%). This is in keeping with the findings discussed in Chapter 3. It is important to note that this study included non-Caucasian patients, from different ethnic groups. There were no *vacA* s2/m1 strains. Eight strains were untypeable for the *vacA* m region and one for the s region. As discussed in chapter 3, this is consistent with the findings of other groups, and may be explained by the existence of greater heterogeneity of the *vacA* mid-region between different geographical groups (Strobel *et al*, 1998; Wang *et al*, 1998).

4.4.2 Bacterial density in association with PUD.

Work by Atherton (Atherton *et al*, 1996) and Khulusi (Khulusi *et al*, 1995) showed an association between density of bacterial colonisation *in vivo* and the presence of ulceration. In this study there was no such convincing relationship between bacterial density and the presence of peptic ulceration ($p=0.8$), when analysed as a whole (PUD) or within the GU and the DU groups. It is important to acknowledge that differences between studies may reflect the difficulties with accurately assessing bacterial density. All three studies had a single blinded

histopathologist assessing the samples. This study assessed density at the antrum only and used only one biopsy sample, compared to the other studies, which histologically assessed both multiple antral and body samples. Although, it is interesting to note that Atherton and colleagues found that antral density was a more reproducible measurement than body density (Atherton *et al*, 1996).

4.4.3 *cagA*, disease, bacterial density and inflammation.

The *cagA* gene acts as a genetic marker for an island of genes that are thought to be responsible for some of the pathogenic mechanisms of *Helicobacter pylori* (Censini *et al*, 1996). This *cag* pathogenicity island encodes several genes, including *cagA* and the genes *picA* and *picB*, which are involved in the enhanced release of the cytokine interleukin 8, and subsequent gastric inflammation and ulceration (Tummuru *et al*, 1995). It has been suggested that *cagA* positive strains may cause extensive inflammation not only by inducing pro-inflammatory cytokines, but also by an increased ability of the bacteria to colonise the gastric mucosa (Atherton *et al*, 1996).

In this study the presence of *cagA* was not significantly associated with the presence of peptic ulcer disease ($p= 0.10$). Infecting *cagA* positive strains were associated with denser colonisation of *H. pylori* and a more marked antral inflammatory response when compared to *cagA* negative strains. When the distribution of strains was analysed within the PUD and the NUD groups, the association of *cagA* with density and chronic inflammation persisted. These findings, in conjunction with other studies (Atherton *et al*, 1996; Peek *et al*, 1995) emphasize

that *cagA* is important in bacterial colonisation and the pathogenesis of inflammation and peptic ulceration.

4.4.4 *vacA*, disease, bacterial density and inflammation.

The *vacA* type s1 was associated with PUD, but 82.4% of patients without active ulcer disease were also infected with *vacA* s1 strains. There was no difference in the distribution of *vacA* types s1/m1 and s1/m2 in the NUD and the PUD groups. Of interest is that there was only one type s2 strain in the PUD group compared with 12 in the NUD group. The presence of *cagA* was significantly associated with *vacA* s1 type ($p<0.05$).

Strains with the type *vacA* s1/m1 (associated with *in vitro* cytotoxin production) and expression of *cagA* have been labeled as type I strains (Xiang et al, 1995). Such strains are expected induced a more marked inflammatory response and ulceration than type II strains (*cagA* negative/ *vacA* s2m2)(Telford *et al*, 1997). Potentially, type I strains induce mucosal damage via cytotoxin production and highly immunogenic proteins (in particular the cytotoxin and the 120-140 kDa product of *cagA*). In this study, a minority of individuals were infected with type I strains, both with (39%) and without (28.4%) peptic ulceration. Thus, the type *cagA* positive/ *vacA* s1 is not a potential candidate as a marker for active ulcer disease in this population. The small number of *cagA* –ve/ s2m2 (type II) strains in this population (20/120), means that a direct comparison between types I and II cannot reliably be made in this study.

There was a significant correlation between *vacA* s1 type and enhanced chronic inflammation, independent of the *cagA* status of the strain. This trend is in keeping with a study in the USA

(Atherton *et al*, 1997) and could be explained by the finding that greater levels of the vacuolating cytotoxin are produced by *vacA* s1 type compared with other *vacA* genotypes (Atherton *et al*, 1995). Thus, we would expect the s1 strains to cause extensive epithelial cell damage, inflammation and potentially ulceration. Once again, the paucity of s2 strains in this study (13/120) renders analysis of type s1 versus type s2 in terms of inflammation and density unreliable.

Analysis of the *vacA* mid region showed there was no correlation with either bacterial density or enhanced inflammation. However, there was an interesting relationship between gastroduodenal ulcers and the *vacA* mid-region in this study. The majority of the gastric ulcer strains were *vacA* m1 compared with the duodenal ulcer strains, which showed a predominance of m2 types. The significance of this is uncertain and further work is needed to confirm the findings.

4.4.5 Criticisms and considerations for future studies.

In this study, histological grading was performed on one antral sample. By relying on grading from only one site (the antrum) and on one sample, the presence of *H. pylori* and the severity of the gastric inflammation may be misrepresented. This point is highlighted by the fact that the grade '0', representing the absence of inflammation and organisms, is noted in several of the patients in whom infection with *H. pylori* has been independently confirmed. By grouping the grading of inflammation and density in terms of mild (0-1) and moderate-severe (2-3), in the majority of cases the data is likely to be correctly represented, but the margin of error will be higher than if several samples were histologically graded.

It is important, as was carried out in this study, that a single experienced Histopathologist, with an interest in Gastroenterology, is responsible for grading the biopsies. In this study, the Histopathologist was blinded to the genotyping, but not the gastroduodenal pathology of the patient. Blinding of the Histopathologist to the pathology, although ideal, is difficult in a clinical setting. For an accurate diagnosis, the histopathologist needs a detailed clinical history and patient details in order to decide on the appropriate histological methodology.

The study shows a correlation between *vacA* s1 types and the severity of inflammation. However, the relevance of *vacA* s1 is only truly appreciated if compared to the alternative genotype s2. This point is important to note, but was not possible to analyse reliably in this study population, where the s2 genotype is so uncommon.

An important and clinically informative question to be answered is if those patients with non-ulcer dyspepsia infected with *cagA* positive strains and with a severe inflammatory response, will go on to develop serious pathology over a period of time (probably decades). Such a study would not only be costly, and complex statistically, but ethically impossible, especially with increasing evidence of disease-association with *H. pylori*.

4.4.6 Summary and significance of the findings

In summary, this work has demonstrated that *cagA+* strains are associated with a dense infiltration of *H. pylori* and an enhanced inflammatory response. This is in keeping with the finding that *cagA* acts as a marker for other genes on the '*cag* pathogenicity island' which are involved in the pathogenesis of inflammation. The significance of the lack of association of bacterial density with peptic ulceration in this study is uncertain, but the low number of antral samples taken for histological assessment is likely to be of importance. *vacA* s1 type is independently associated with increased inflammation and gastroduodenal ulceration, but there was no correlation with density of bacterial colonisation.

As with the previous study (Chapter 3), certain points are highlighted regarding strain type and pathology. The presence of *cagA* was associated with *vacA* s1. Although *cagA* and the *vacA* s1 allele were associated with the presence of PUD, the *cagA* positivity rate and *vacA* s1 types in patients without PUD was also high (70.3% and 82.4% respectively). This implies that the presence of a *cagA* and/ or *vacA* s1 cannot reliably predict the presence of active ulcer disease. The untypeable m regions of 8 strains is in keeping with the accumulating evidence that there is greater diversity of the mid-region than initially thought.

Chapter 5

CagA protein, VacA cytotoxin, serological response and disease.

ABSTRACT

Background- Detection of anti-CagA and anti-VacA antibodies has been associated with gastroduodenal pathology, and the presence of neutralising anti-VacA antibodies indicates that the mature toxin is expressed *in vivo.* CagA protein size varies between 120-140 kDa, but it is uncertain whether size variation is of clinical relevance.

Objective- To look at the relationship between size of CagA and pathology. To determine if serological detection of antibodies to the cytotoxin and the CagA protein correlate with macroscopic and microscopic pathology and *in vivo* strain *vacA* type and *cagA* status.

Methods- The molecular size of CagA was determined in 62 dyspeptic patients by immunoblotting against biotinylated weight standards, and the results correlated with pathology. Anti-VacA and anti-CagA antibodies were detected using a commercial immunoblot, Helicoblot 2.0, in 48 patient samples. The *vacA* s and m types and the *cagA* status were determined by PCR and analysed in relation to the presence of antibodies.

Results- Analysis of CagA size showed no association with PUD or grade of inflammation/ bacterial density, but in the three gastric cancer patients the median and mean molecular weight (135.5 and 136 respectively) were higher than in patients with PUD/ gastritis. Antibodies to VacA were detectable in only 17/ 38 patients infected with *vacA* s1 type. All 3 patients infected with *vacA* s2 strains had no detectable anti-VacA antibodies. 71.4% of PUD and 40% of NUD serums had detectable anti-VacA antibodies but this did not reach statistical significance (p=0.061). In 4/ 48 patients, there was a discrepancy between the *cagA* status and the presence of anti-CagA anti-bodies. All 6 gastric cancer patients had antibodies to CagA. Twenty one of the 26 PUD patients and 12/ 16 NUD patients had detectable antibodies to CagA (p=0.471).

Discussion- There was no associated between PUD and detectable anti-VacA or anti-CagA antibodies. There was no association between *in vivo vacA* s1 type and the presence of antibodies to the vacuolating cytotoxin. The higher molecular weight CagA proteins were associated with gastric cancer, but the sample size was small (3).

5.1 Introduction.

The vacuolating cytotoxin, encoded by the *vacA* gene, is expressed in only 50% of *H. pylori* strains (Leunk *et al*, 1988). Specific *vacA* genotypes correlate with *in vitro* cytotoxin activity, with *vacA* s1/m1 type producing the greatest levels of cytotoxin and s2/m2 the least (Atherton *et al*, 1995). Detection of neutralising anti-VacA antibodies in the serum of infected individuals indicates that the mature cytotoxin is expressed *in vivo* (Cover *et al*, 1992).

The *cagA* gene encodes a highly immunogenic protein CagA, with a molecular weight of 120-140 kDa. The size variation of CagA is thought to be due to the presence of a variable number of repeat sequences located within the 3' region of the gene (Covacci *et al*, 1993). The importance of the repeat sequences and CagA size variability is uncertain, but a recent study has shown that the primary gene structure of the 3' region of *H. pylori* isolates in Japan, differ markedly from that of Western isolates, and that structural subtypes of the 3' region are responsible for variation in CagA size (Yamaoka *et al*, 1998).

5.1.1 Association between CagA size and microscopic and macroscopic pathology.

The importance of CagA size remains largely unknown. An Italian group noted that CagA proteins with a higher molecular weight tended to be associated with gastric cancer (Sepulveda *et al*, 1997). More recently, a study in Japan showed that CagA size is determined by the number and configuration of repeat sequences in the 3' region of the *cagA* gene, with a specific structural subtype of the region resulting in proteins with higher molecular weights, which were associated with gastric cancer (Yamaoka *et al*, 1998). The association of CagA size and pathology has not been studied in a UK dyspeptic population.

5.1.2 Detection of anti-CagA and anti-VacA antibodies.

Multiple studies have used serological detection of antibodies to the CagA protein and the vacuolating cytotoxin as a means of looking at the association between these major virulence factors and gastroduodenal pathology (Maeda *et al*, 1998; Rudi *et al*, 1997; Xiang *et al*, 1995). In fact, the initial identification of CagA was first noted serologically (Crabtree *et al*, 1991). An increasing number of commercially available serological kits are becoming available for detection of these antibodies, with a view to screening dyspeptic populations and identifying individuals most at risk of developing *H. pylori* related pathology. The reliability and accuracy of using such kits as a means of diagnosing ' at risk individuals ' in the clinical setting remains uncertain.

5.1.3 Association of disease, *in vivo vacA* subtype and *cagA* status, and serological detection of anti-VacA and anti-CagA antibodies.

Expression of cytotoxin activity in different *H. pylori vacA* subtypes has been characterised by *in vitro* studies of vacuolation and cell damage (Atherton *et al*, 1995). In contrast, i*n vivo* expression of the vacuolating cytotoxin can be studied by serological detection of anti-VacA antibodies (Cover *et al*, 1992). The association between *in vivo vacA* s and m type, *in vivo* expression of cytotoxin and disease type needs to be clarified. Similarly, *in vivo* expression of CagA can be studied, and correlated with *in vivo* typing of the *cagA* gene.

5.1.4 Aims to be tested.

The aims of this study were:

1. to look at the association between CagA size and gastroduodenal pathology

2. to compare detection of the CagA protein using immunoblotting with direct protein staining

3. to compare the presence of serum anti-VacA and anti-CagA antibodies with the *in vivo* strain type and pathology.

5.2 Methods.

Patient selection and clinical assessment (section 2.2), bacterial culture and antigen preparation (section 2.5) and DNA preparation (section 2.6) are detailed in Chapter 2.

5.2.1 PCR typing

The *cagA* status and the *vacA* s and m types of infecting strains were typed by PCR as described in chapter 2 (section 2.7)

5.2.2 Histological assessment

Histological assessment was performed, and grading of inflammation and density of bacteria carried out according to the Sydney system (Dixon *et al*, 1996). Grading of density and inflammation were grouped as grades 0-1 and 2-3, as described in the method section of Chapter 4 (4.2.1).

5.2.3 Serum analysis

The 48/ 61 dyspeptic patients previously described (3.3.1), had serum samples taken at the time of the endoscopy procedure. The presence of anti-CagA and anti-VacA antibodies were detected using Western blotting and a commercial Western blot, Helicoblot 2.0. Both methods are described in detail in sections 2.9.

5.2.4 Molecular weight estimation of the CagA protein

The molecular weight of the CagA protein was estimated by SDS-PAGE followed by immunoblotting with an anti-CagA antibody positive serum, against biotinylated molecular weight standards.

The CagA protein was visualised by immunodetection with a known anti-CagA antibody positive human serum sample, diluted 1/5000 and an anti-human IgG horseradish peroxidase conjugated diluted 1/2500. The biotinylated molecular weight standards were revealed by inclusion of a streptavidin-horseradish peroxidase conjugate diluted 1/3000 in the secondary antibody buffer. Band development was achieved using ECL as described previously.

The CagA molecular weight estimation was carried out using Image Master™ 1-D (one-dimensional) gel analysis software (version 1.20; Pharmacia Biotech) against a standard curve determined from the molecular weight standards.

Silver staining of 26 bacterial antigen preparations (as detailed in Chapter 2, section 2.8.2) was carried out and compared with the results of immunoblotting.

5.3 Results

5.3.1 CagA protein size and association with microscopic and macroscopic pathology.

5.3.1.1 Patient characteristics

Strains were successfully cultured from 62 symptomatic patients. Thirty four patients had NUD, 25 PUD (19 DU and 6 GU), and 3 had gastric cancer.

5.3.1.2 CagA size and gastroduodenal pathology

The molecular weight of the CagA protein was analysed in strains from patients with different gastroduodenal pathology, and is detailed in tables 5.1.

Of the 34 strains from patients with NUD, the mean and median molecular weight of the CagA protein were 131.8 and 130 respectively, with a range of 124 – 143. The mean and median values of CagA size in the 25 PUD strains were not appreciably different compared to the values of the NUD strains: a mean of 128 and median of 128.5 in GU strains and a mean of 131.9 and median of 131 in DU strains.

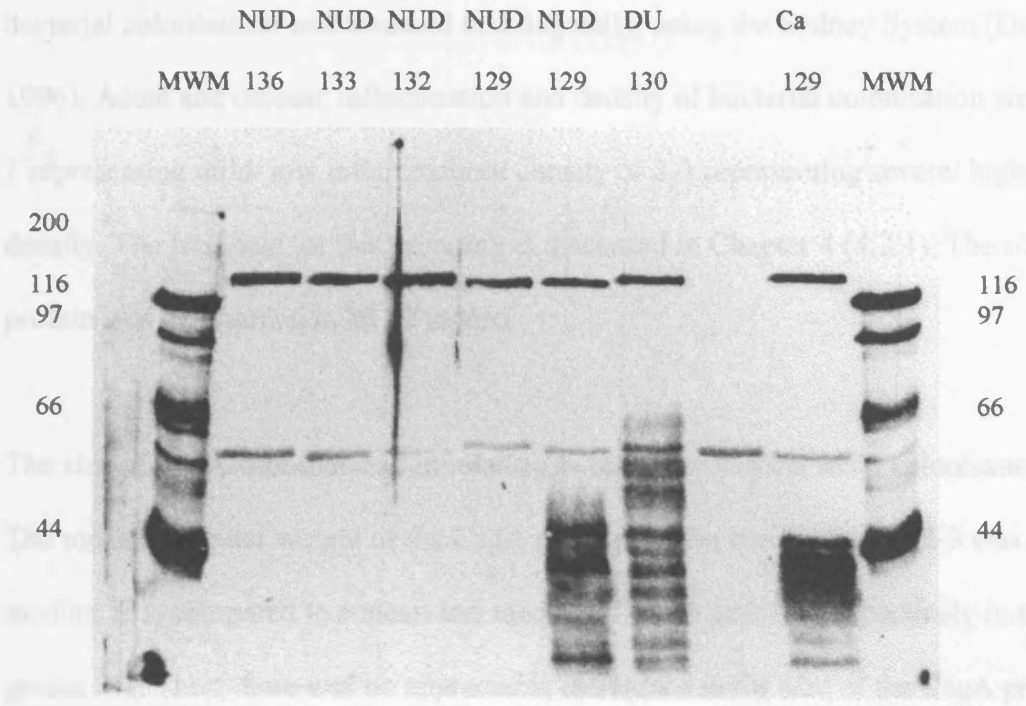
The median (136) and mean (135.5) CagA weights were higher in the gastric cancer isolates than in isolates from the other disease groups. However, *H. pylori* isolates were cultured from only three gastric cancer patients.

Table 5.1- CagA molecular weight size (kDa), according to pathology

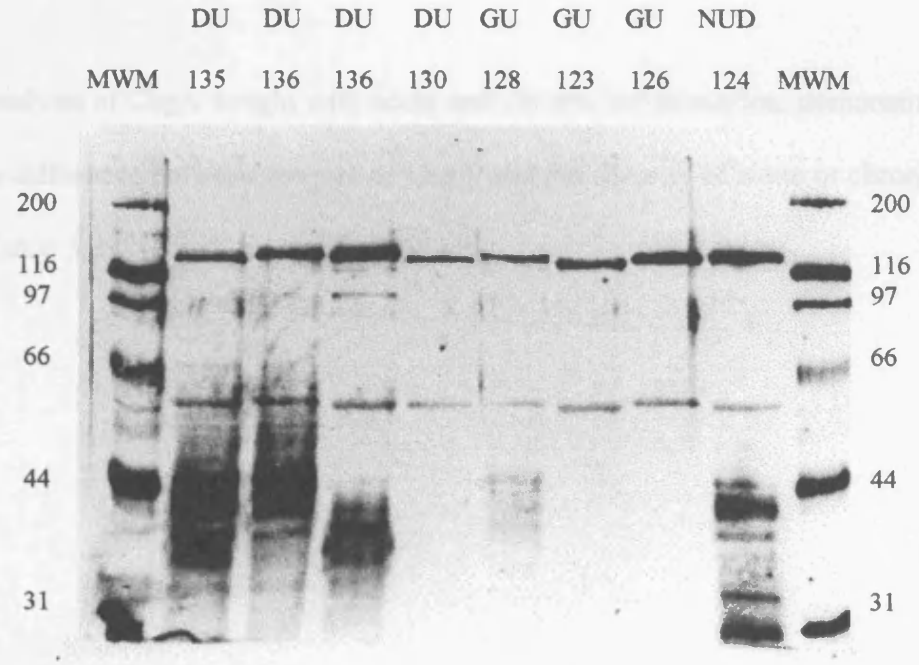
NUD - CagA size	DU - CagA size	GU – CagA size	Ca - CagA size
124	124	123	133
128	127	126	136
128	128	128	137
128	128	129	
128	129	131	
128	130	131	
128	130		
128	130		
129	130		
129	131		
129	131		
129	133		
130	133		
130	134		
130	135		
130	136		
130	136		
131	137		
132	144		
132			
132			
132			
132			
133			
133			
136			
136			
136			
137			
137			
137			
137			
138			
143			
MEAN – 131.8	MEAN – 131.9	MEAN - 128	MEAN – 135.5
MEDIAN - 130	MEDIAN - 131	MEDIAN - 128.5	MEDIAN – 136

Figures 5.1 and 5.2: CagA size variation using ECL detection in a series of patients.

Figure 5.1



Figures 5.2



5.3.1.3 CagA size, inflammation and bacterial density.

Strains were cultured from 42 patients in whom acute and chronic inflammation and density of bacterial colonisation was assessed histologically, using the Sydney System (Dixon *et al*, 1996). Acute and chronic inflammation and density of bacterial colonisation were graded as 0-1 representing mild/ low inflammation/ density or 2-3 representing severe/ high inflammation/ density. The rationale for this grouping is discussed in Chapter 4 (4.2.1). The size of the CagA protein was determined in all 42 strains.

The size of CagA was analysed in relation to the density of bacterial colonisation (Table 5.2). The mean molecular weight of the CagA proteins in the density grades 2-3 was 132.5 and the median 131, compared to a mean and median of 129.9 and 129 respectively in the density grades 0-1. Thus, there was no appreciable difference in the size of the CagA protein and the density of bacterial colonisation.

Analysis of CagA weight with acute and chronic inflammation, demonstrated that there was no difference between the size of CagA and the severity of acute or chronic inflammation (Table 5.2)

Table 5.2 Analysis of the CagA molecular weight size in relation to bacterial density and chronic and acute inflammation.

Histological grade		Mean MWt	Median MWt	Range of MWt
Density	0-1	129.9	129	124 - 137
	2-3	132.5	131	128 - 144
AI	0-1	129.7	129	122 - 137
	2-3	133.6	131	124 –145
CI	0-1	128	128.5	126 – 129
	2-3	132.1	130.5	122 - 145

MWt = molecular weight size

CI = chronic inflammation

AI = acute inflammation

5.3.2 Detection of bacterial CagA protein.

Twenty six of the 31 strains isolated from the 61 dyspeptic patients discussed in Chapter 3 were freshly cultured on Chocolate agar as described previously. Bacterial antigen preparations were fractionated and separated by SDS-PAGE, and bacterial proteins visualised using silver staining technique (as described in section 2.8.2) and immunoblotting, using an anti-CagA positive serum as the primary antibody. A negative control sample was prepared from a Chocolate agar plate without bacteria to provide a sample of plate protein (figures 5.9 and 5.10).

A comparison between detection of the CagA protein by silver staining and by immunoblotting was made in the different disease groups. There were no discrepancies between the two methods of CagA protein detection.

Five strains cultured from patients with DU were CagA positive. Figure 5.3 and 5.4 show the results of detection of CagA, using silver staining and immunoblotting respectively.

Figure 5.3 Silver stain SDS-PAGE gel of strains from patients with DU.

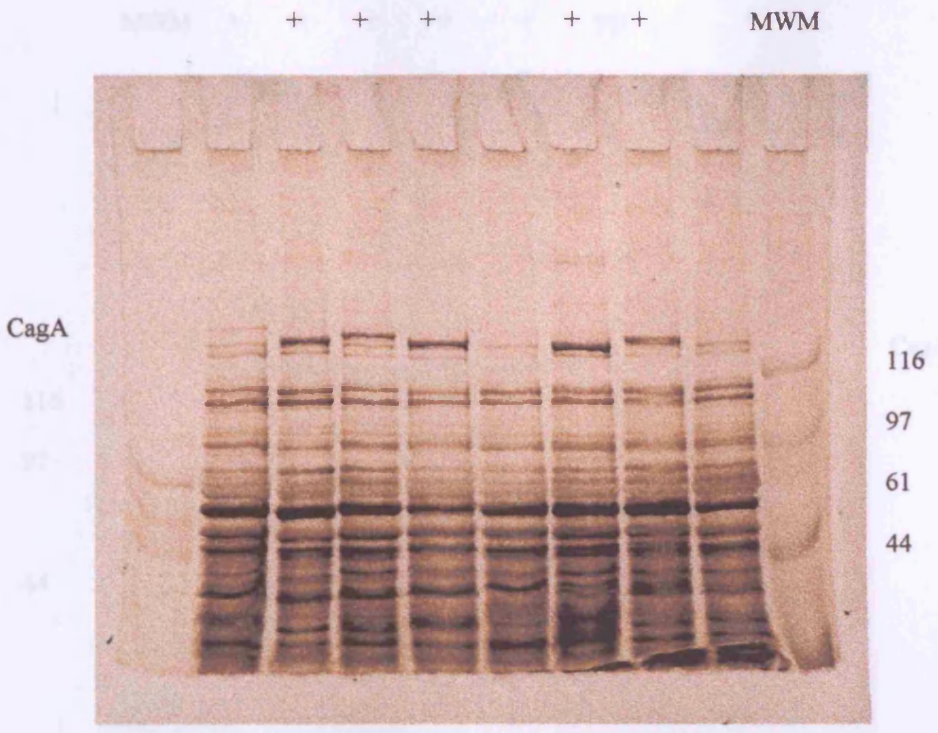
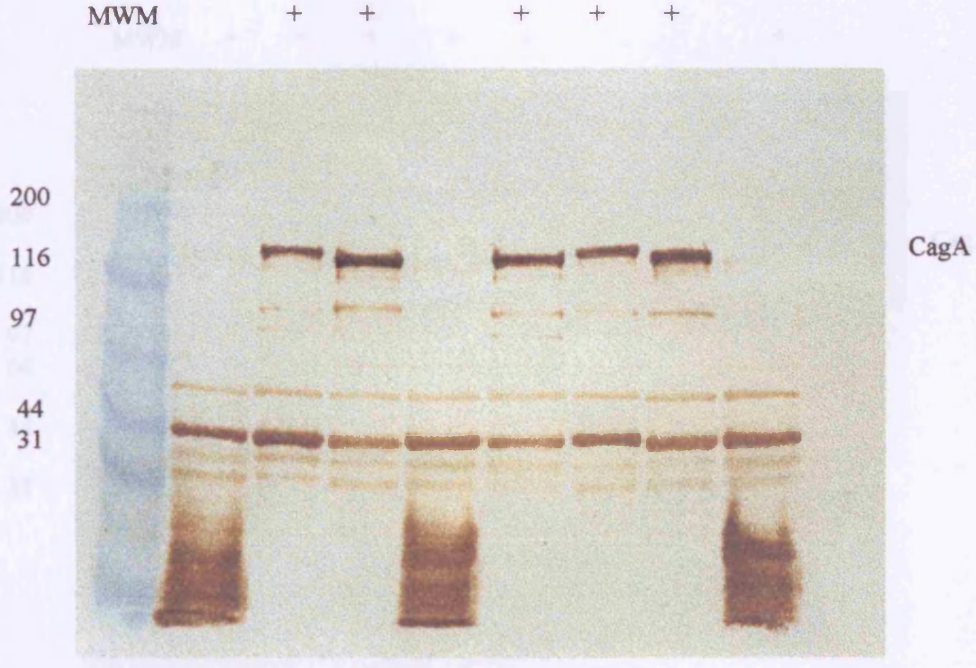


Figure 5.4 Immunoblot gel of CagA in the same DU strains.



Figures 5.5 Silver stain SDS-PAGE gel of GU and gastric cancer strains, (pp = plate proteins).

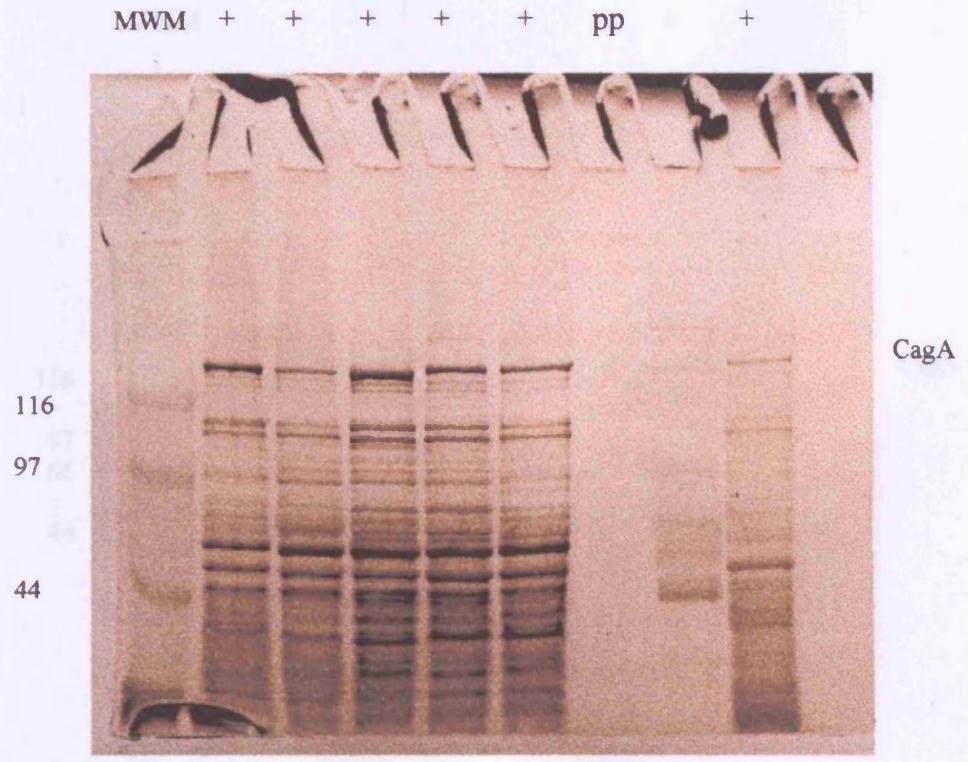


Figure 5.6 Immunoblot gel of the same strains from patients with GU and gastric cancer

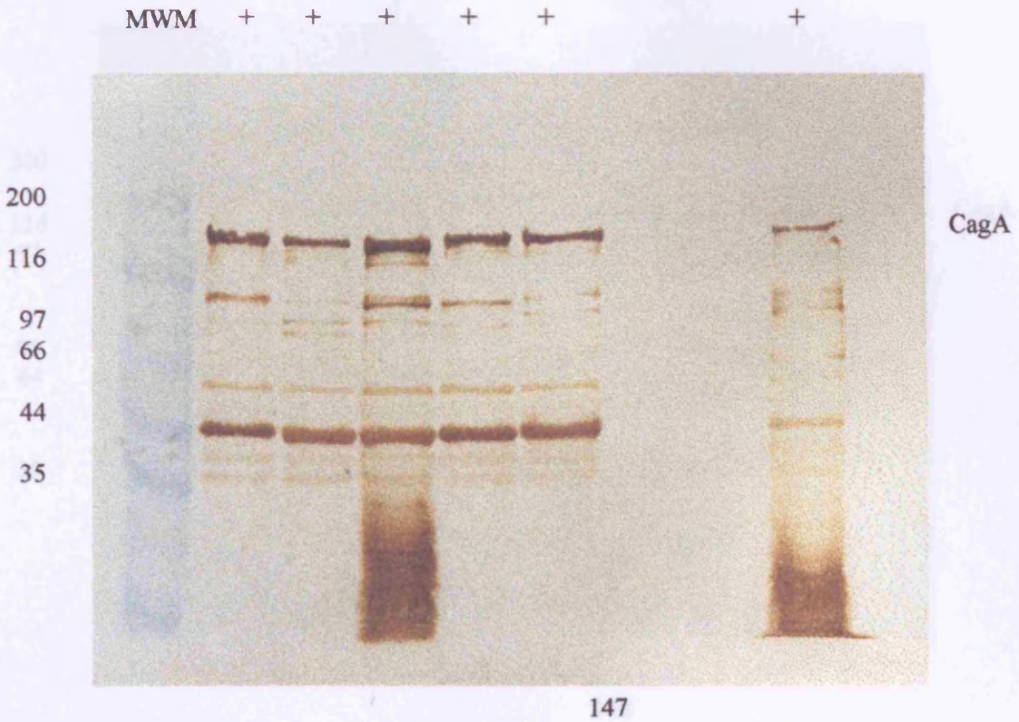


Figure 5.7 Silver stained SDS-PAGE gel of 9 strains from NUD patients

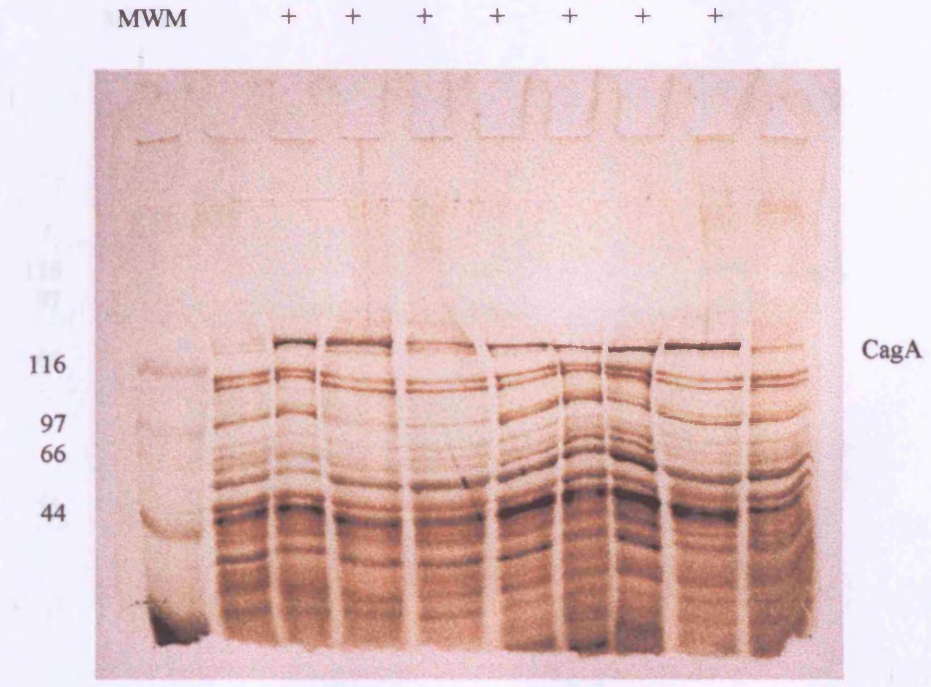


Figure 5.8 Immunoblot gel of NUD strains in the same series of patients

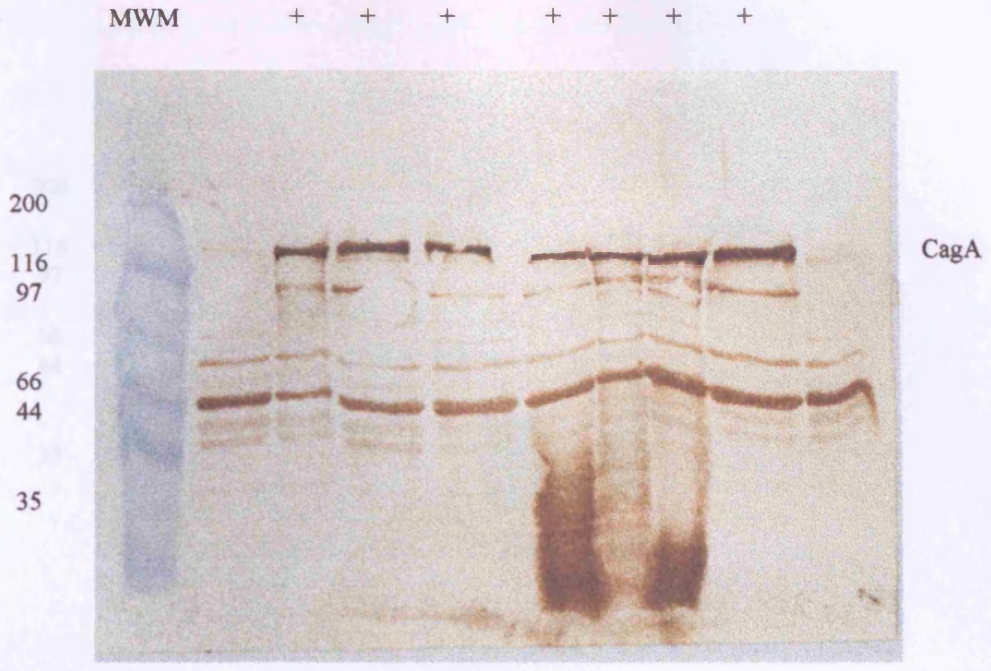


Figure 5.9 Silver stain SDS-PAGE gel of 8 CagA negative strains and a positive control

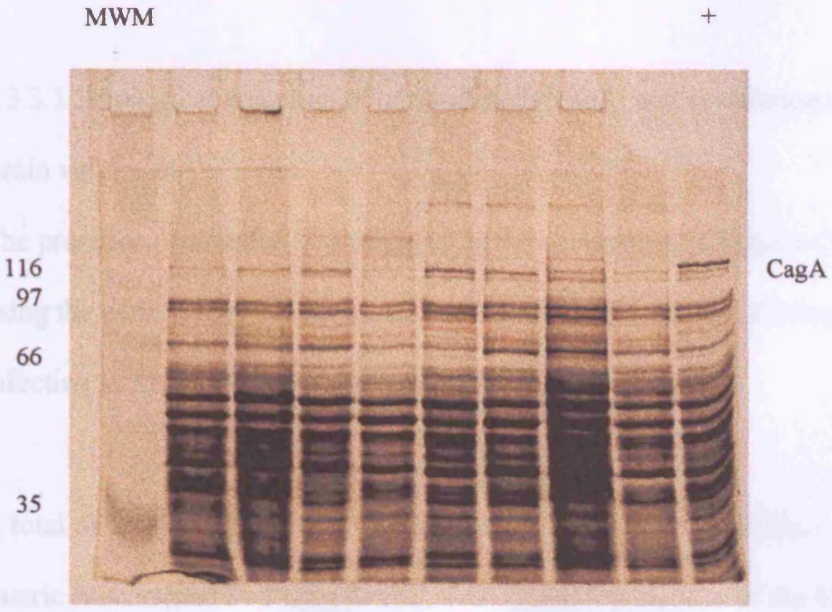
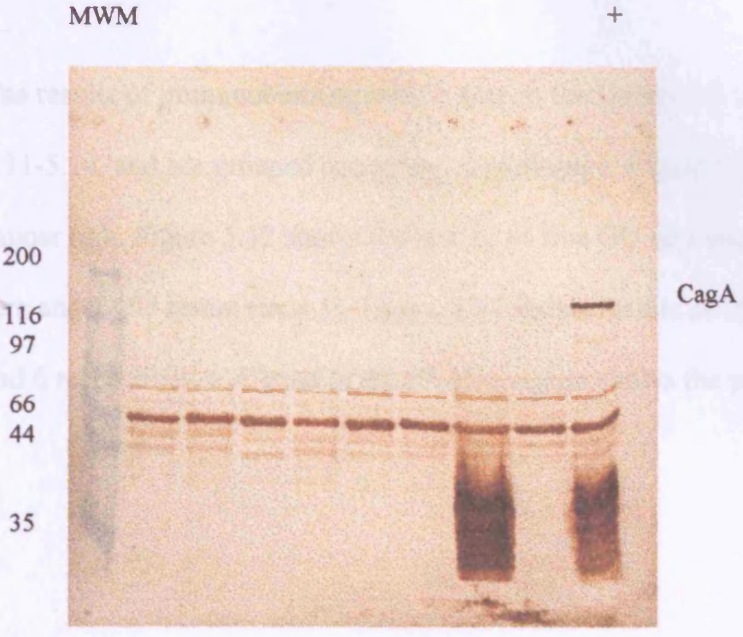


Figure 5.10 Equivalent immunoblot



5.3.3 Serology data for detection of the CagA protein and VacA.

5.3.3.1 Serological detection of antibodies to VacA, and correlation with *in vivo* typing of strain *vacA* s and m types.

The presence or absence of antibodies to the vacuolating cytotoxin (VacA) were detected using the commercial Western blot kit, Helico-2-blot, and the findings correlated with the infecting strain *vacA* type as defined by PCR DNA typing.

A total of 41/ 48 patients were studied for the presence of anti-VacA antibodies: 15 NUD, 5 gastric cancers and 21 PUD (14 DU, 7 GU). Interestingly, 4 of the 41 (9.75%) patients were *H. pylori* positive as defined by CLO, histology and PCR, but negative for anti-*H. pylori* antibodies.

The results of immunoblotting patient sera in the Helicoblot 2.0 system are shown in figures 5.11-5.14, and are grouped according to pathology. Figure 5.11 shows the results of 5 gastric cancer sera. Figure 5.12 shows the results of five GU sera and figure 5.13 the results of 13 DU sera and 1 GU serum (lane 1). Figure 5.14 shows results of 15 NUD, 1 GU and 1 DU (lanes 5 and 6 respectively). A band in the 89-kDa region shows the presence of antibodies to VacA.

Figure 5.11 Helicoblot 2.0 detection of anti-VacA antibodies in 5 gastric cancer patients

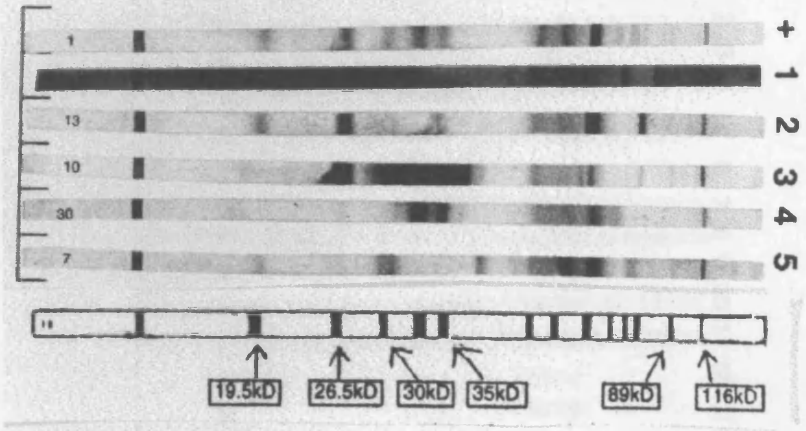


Figure 5.12 Helicoblot 2.0 detection of anti-VacA antibodies in 5 GU patients

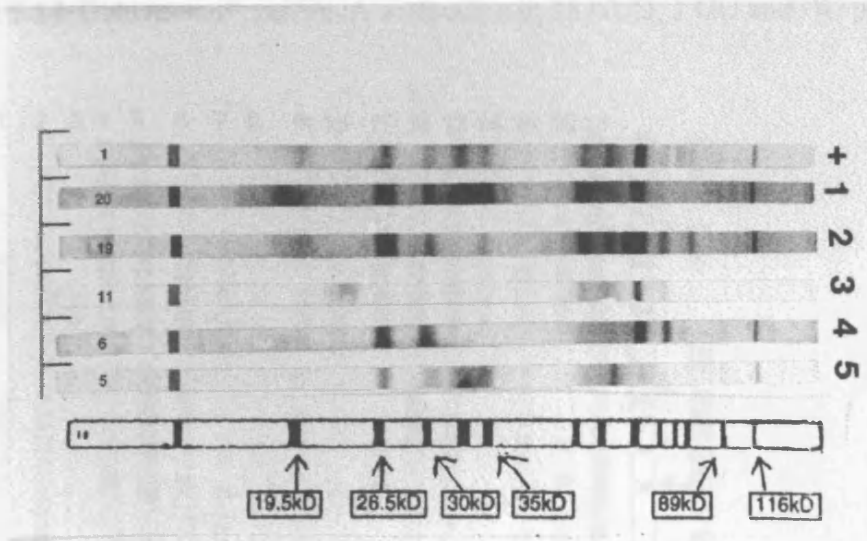


Figure 5.13 Helicoblot 2.0 detection of anti-VacA antibodies in 13 DU and 1 GU patients

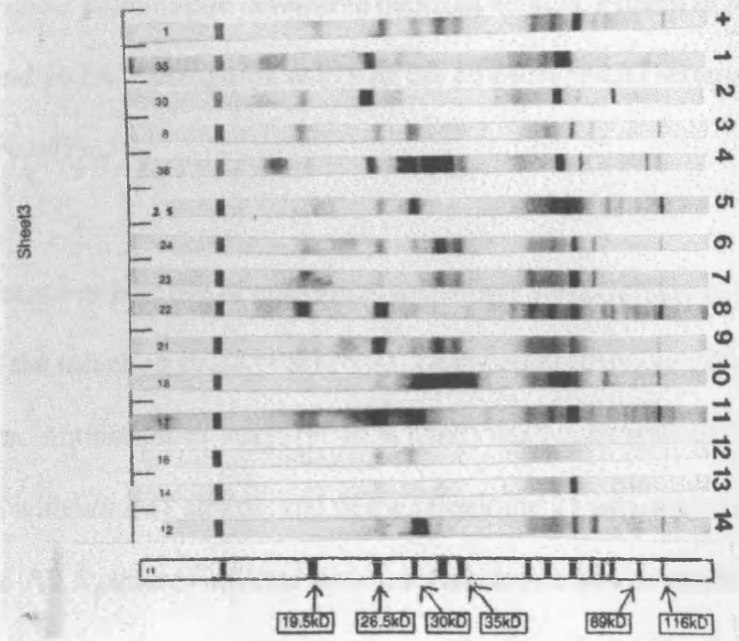
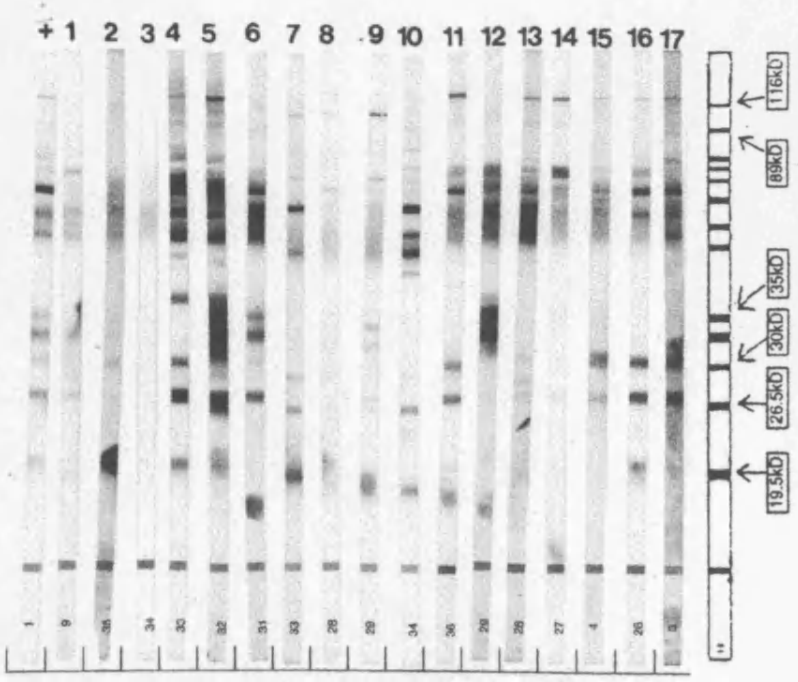


Figure 5.14 Detection of anti-VacA antibodies in 15 NUD, 1 GU and DU patients



A total of 24/ 41 (58.5%) patient sera had detectable anti-VacA antibodies. Three of the 5 gastric cancer patients had detectable bands at 89 kDa. Fifteen of the 21 (71.4%) PUD serums (5 GU and 10 DU), compared with 6 of the 15 (40%) NUD serums, had detectable bands at 89 kDa (p=0.061).

The presence or absence of anti-VacA antibodies was analysed in relation to the *vacA* s and m types of the infecting strain (Table 5.3). Thirty eight of the 41 patients were infected with *vacA* s1 strains. Antibodies to VacA (89 kDa band) were detectable in 17 of the 38 (44.7%) patients infected with *vacA* s1 strains, and in the remaining 21 patients anti-VacA antibodies were not detected. All 3 patients infected with *vacA* s2 strains had no antibody to the cytotoxin. There was no difference between the presence or absence of anti-VacA antibodies and *vacA* m1 and m2 types (p=0.50).

Table 5.3 Association of anti-VacA antibodies with the *vacA* s and m types of the infecting strain.

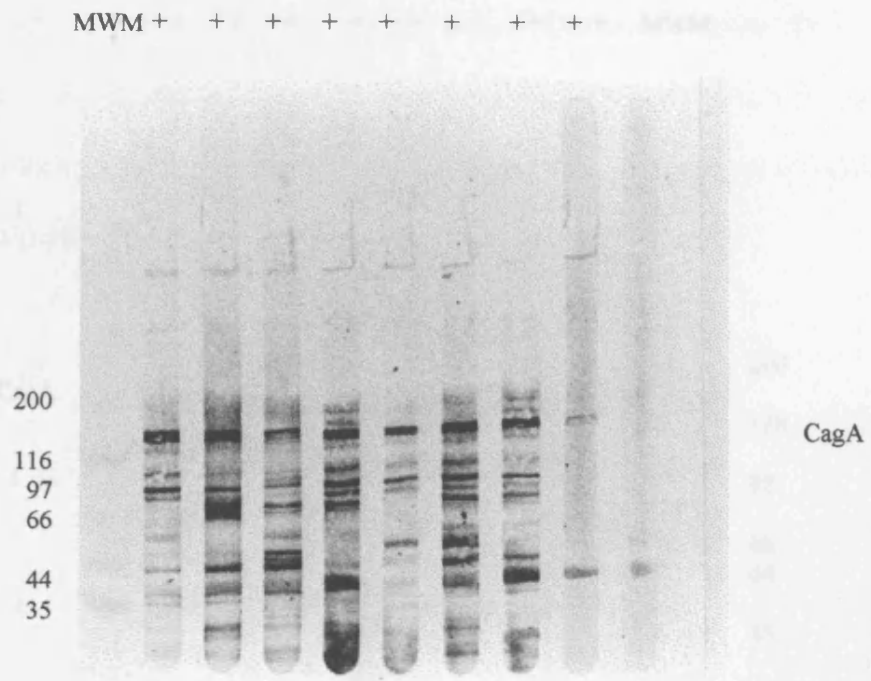
Anti-VacA antibodies (89 kDa)	*vacA* s1	*vacA* s2	*vacA* m1	*vacA* m2	No m type
Present	17	0	6	9	2
Absent	21	3	8	15	1
Total	38	3	14	24	3

5.3.3.2 Serological detection of antibodies to CagA, and correlation with *in vivo* typing of strain *cagA* status.

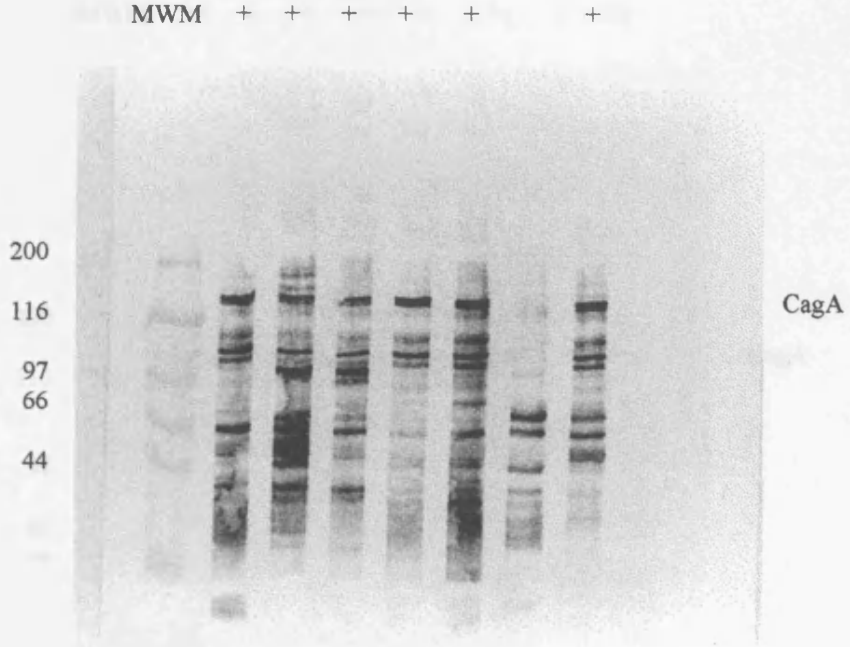
Forty eight serum samples were tested for anti-CagA antibodies by immunoblotting against an ultracentrifuged whole cell-sonicate antigen of the *cagA* positive strain *H. pylori* NCTC 11637. The antigen was fractionated on 7.5% polyacrylamide gels using the Laemmeli (Laemmeli, 1970) buffer system and transferred to Hybond-C super membranes as described in chapter 2, section 2.9. Detection of anti-CagA antibodies is described in section 2.9.

A total of 39/ 48 (81.2%) samples had detectable anti-CagA antibodies. In patients with detectable antibodies to VacA, anti-CagA antibodies were also present. The presence of antibodies to CagA was analysed in relation to pathology. All 6 gastric cancer patients had detectable anti-CagA antibodies (figure 5.15, tracks 1-6). A total of 26 PUD patients were analysed, and anti-CagA antibodies were detectable in 21/ 26 serum samples (80.8%). Seven of the 8 GU (figure 5.16) and 15/ 18 DU serum samples had detectable anti-CagA antibodies. An immunoblot of 9 DU serum samples is shown in figure 5.17. Twelve of the 16 NUD patients (75%) had antibodies to CagA (figure 5.18 shows the results of an immunoblot of 8 of the NUD serum samples). There was no association of PUD with the presence of anti-CagA antibodies ($p=0.471$).

Figures 5.15 Immunoblot gel of anti-CagA antibodies in gastric cancer patients



Figures 5.16 Immunoblot gel of anti-CagA antibodies in 7 of the 8 GU patients



Figures 5.17 Immunodetection of anti-CagA antibody in 9 DU patients

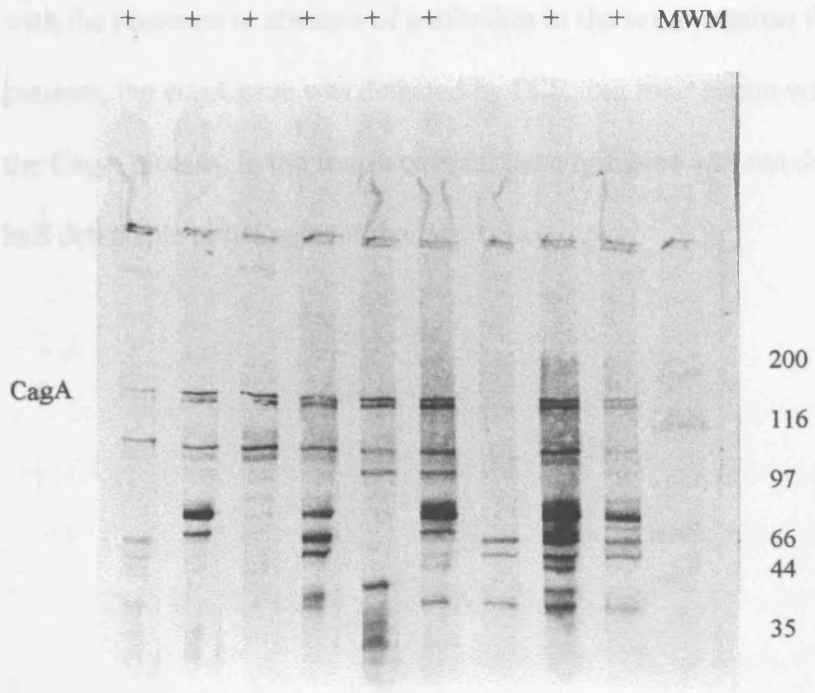
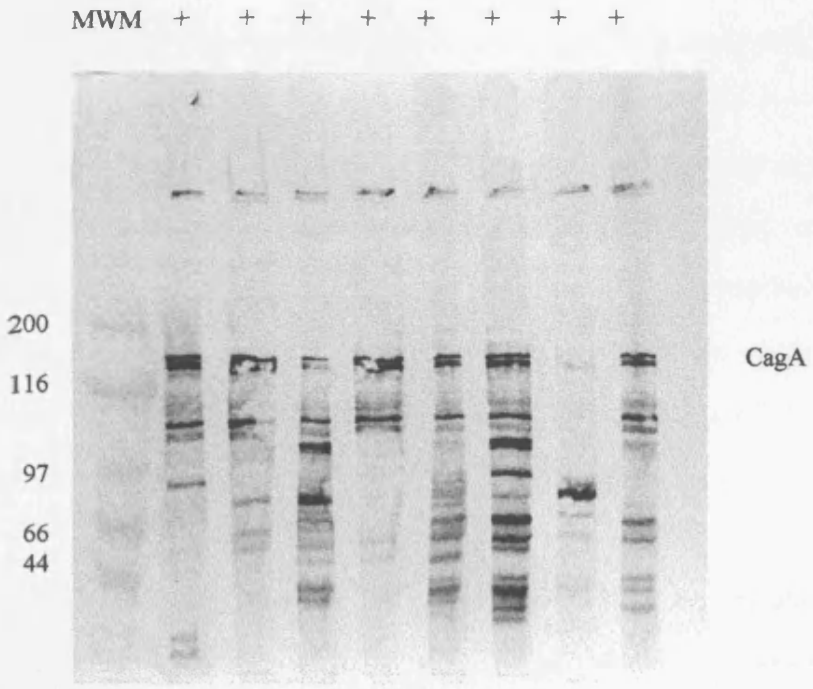


Figure 5.18 Immunodetection of anti-CagA antibody in 12 NUD patients



In 44/ 48 patients, the *cagA* status, as defined by PCR amplification of biopsy DNA, correlated with the presence or absence of antibodies in the serum against the CagA protein. In 3 patients, the *cagA* gene was detected by PCR, but their serum was negative for antibodies to the CagA protein. In the fourth patient, the *cagA* gene was not detected by PCR but the patient had detectable anti-CagA antibodies.

5.4.4 Discussion

This work looked at the use of serological analysis in a dyspeptic population as a means of detecting *H. pylori* infection, and compared the *in vivo* expression of the cytotoxin and CagA proteins with *in vivo* strain type and pathology. The association of CagA size with pathology was studied.

5.4.1 CagA size and gastroduodenal pathology.

The molecular weight size of the CagA protein was determined in 62 isolates from dyspeptic patients, with a variety of pathologies. In 26 of the isolates, the detection of CagA by immunoblotting was compared with direct detection of CagA protein using silver staining. The purpose of including this data was to ensure that the immunoblotting technique was successfully detecting the CagA protein. There was no discrepancy between the two methods.

The range of molecular weights varied from 123-144 kDa. No association was demonstrated between size of CagA and the presence of PUD, grade of gastric inflammation or bacterial density. Both the mean and the median of CagA size were higher in the isolates from patients with gastric carcinoma, but the numbers (3) are too small for any conclusion to be drawn from this data. This trend, however, has been noted by two other groups. Sepulveda and colleagues found that 50% of the strains isolated from patients with gastric cancer had a molecular weight of greater than 128 kDa, compared to 6% of the isolates from patients with duodenal ulcers or gastritis (Sepulveda *et al*, 1997). In a detailed study carried out in Japan, isolates with CagA molecular weights of 141-142 kDa were disproportionally high in patients with gastric cancer compared to patients with duodenal ulcers/ gastritis (Yamaoka *et al*, 1998).

The function of the CagA protein is not known, and it is certainly of interest that the higher molecular weight CagA proteins in three separate geographical study populations tend to be

associated with gastric cancer. Variation of CagA size has been correlated with the presence of a variable number of repeat sequences in the 3' region of the *cagA* gene (Covacci *et al*, 1993). Yamaoka and colleagues have shown that in Japanese isolates there are four different types of the *cagA* gene as defined by the number and configuration of repeat sequences in the 3' region, and that the size of the CagA protein is determined by the repeat regions (Yamaoka *et al*, 1998). Interestingly, one of the major repeat sequences differed from those of Western isolates. We were unable to carry out a similar analysis of our UK isolates due to lack of local facilities and expertise, but this would be interesting further work, with a view to comparing the UK and Japanese isolates.

The mechanism behind the association of higher molecular weight CagA proteins with gastric cancer has not been defined, although several suggestions have been considered, one suggestion being that a more marked immune response may be triggered (Yamaoka *et al*, 1998).

5.4.2 Association of anti-VacA antibodies with disease and *vacA* s and m types of the infecting strain.

In this study, 10% of patients were infected with *H. pylori*, as defined by PCR, histology and urease test, but had no detectable anti-*H. pylori* antibodies on immunoblot. Thus, this commercial kit, Helicoblot 2, incorrectly diagnosed 10% of this study population as *H. pylori* negative. This is in keeping with discrepancies demonstrated by other groups between actual sensitivity and specificity values and those quoted by the manufacturers (Schembri et al, 1993). The discrepancy is important, particularly if commercial serological kits are to be used as a means of identifying patients who are suitable for endoscopy, or treatment. It emphases that use of such kits should first be validated for use on the local population as marked

variation between populations can exist, particularly where there is strong ethnic influence (Nair *et al*, 1995).

Fifty nine percent of patients had detectable antibodies to the cytotoxin. Seventy one percent of patients with PUD, compared with 40% of NUD patients, had detectable anti-VacA antibodies, but the association was not statistically significant (p=0.061). There was no association between the *vacA* s type of the infecting strain and the presence of antibodies to the vacuolating cytotoxin, with less than half (44.7%) of the patients infected with s1 type having detectable anti-VacA antibodies. This is an unexpected finding, as *in vitro* studies have shown that the s1 allele is associated with cytotoxin expression and activity (Atherton *et al*, 1995). However, production of the cytotoxin does not directly correlate with cytotoxin activity (as defined by ability of the cytotoxin to induce vacuolation of Hela cells *in vitro*) and thus could explain this discrepancy, although higher concentrations of VacA have been associated with toxigenic strains (Forsyth MH *et al*, 1998). In addition, this study assumes that the vacuolating cytotoxin always produces a serologically detectable antibody, but the immunological response of a patient to an infection is influenced by many host and bacterial factors. To help clarify the relationship between the serological production of antibodies to the cytotoxin with *in-vitro* vacuolation activity of the cytotoxin, *in vitro* studies would need to be carried out. Interestingly, the lack of detectable serological cytotoxin antibodies in patients infected with s2 strains is in-keeping with the *in-vitro* studies (Atherton *et al*, 1995), although the numbers (3) are small. The *vacA* m type had no association with the presence of antibodies to the vacuolating cytotoxin.

In all 24 patients with detectable anti-VacA antibodies, antibodies to CagA were also detected. This finding is in agreement with the association of *cagA* with expression of the vacuolating cytotoxin (Cover *et al*, 1990; Tummuru *et al*, 1993).

5.4.3 Correlation between *cagA* status, pathology and detection of anti-CagA antibodies.

Antibodies to the CagA protein were detected in the majority of patients (81%), and there was no significant association between PUD and the presence of anti-CagA antibodies ($p= 0.471$). The gene *cagA* encodes the highly immunogenic protein CagA (Tummuru *et al*, 1993), and when present it is nearly always expressed. In this study, there was an 8% discrepancy between the presence of *cagA* and the detection of the anti-CagA antibody. In three of the four cases, the *cagA* gene was detected by PCR but no antibody response to the protein was detected. There are a number of possible explanations for these cases. For example, the host may have been unable to produce an immune response to CagA (Glupczynski *et al*, 1992) or the *cagA* gene may not have been expressed *in vivo*. Alternatively, infection could be recent and an immune response may not have yet developed. Finally, technical problems such as false positive PCR results or false negative immunoblot results may explain the discrepancies. In one patient with serologically detectable anti-CagA antibodies, the *cagA* gene was not detected by PCR. This could be accounted for by a false negative PCR result, or that the infection had been cleared or replaced by a *cagA* negative strain, but anti-CagA antibody persisted (Kosunen *et al*, 1992).

5.4.5 Criticisms and future considerations.

Important potential limitations of the methods used in this work need to be considered. The use of SDS polyacrylamide gel electrophoresis to determine the molecular weight of a protein is a relatively easy, rapid and reproducible method and results in the molecular range of 15,000-200,000 are generally within 10% of those obtained by other methods (Weber *et al*, 1975). However, there are several factors which potentially may influence the apparent molecular weight of a protein by altering the electrophoretic mobility and should be considered in this work. The SDS binding can be influenced by the primary structure of a protein, with decreased binding leading to an increased apparent molecular weight. Proteolytic degradation, net charge of the protein and conformational changes can all affect the electrophoretic mobility of some proteins (Weber *et al*, 1975). Ideally, other methods for determination of molecular weight size could have been carried out in addition to the SDS gel electrophoresis for comparison.

Based on the findings of this study, further work is needed. Analysis of repeat sequences in the variable 3' region of the *cagA* gene in the strains isolated in this work, could provide an interesting comparison between the strains of this UK population and the strains of the Japanese study discussed previously (Yamaoka *et al*, 1998). This would provide useful information for future work with these isolates. In addition, it would have been useful to have data on the *in vitro* cytotoxin activity of our strains. This would have enabled comparisons between *in vivo* expression of cytotoxin and CagA protein, as represented by the detection of antibodies, and *in vitro* activity of the cytotoxin.

5.4.4 Summary and significance of the findings

Several important points have been illustrated by this work. Using anti-VacA antibodies to detect *in vivo* expression of the vacuolating cytotoxin, there was no association between infecting strain *vacA* s1 type and *in vivo* expression of cytotoxin. The presence of anti-VacA and/ or anti-CagA antibodies did not significantly correlate with PUD. However, all patients with anti-VacA antibodies also had anti-CagA antibodies, indicating the strong association between expression of vacuolating cytotoxin and the presence of CagA (Tummuru et al, 1993).

The use of serological tests for diagnostic targeting of patients who potentially are at an increased risk of *H. pylori* related pathology, although attractive in simplicity and ease, has been demonstrated in this study population to be unreliable. Validation for use on the local population is essential.

Chapter 6

Ischaemic Heart Disease and CagA

Abstract

Background Previous studies investigating the association of *H. pylori* infection with coronary heart disease (CHD) have produced conflicting results. This study examined whether the genetic diversity of *H. pylori* influenced the association and specifically whether the risk was confined to infection with the more virulent strains bearing the *cagA* gene.

Methods Serological status for *cagA* and *H. pylori* were determined in 342 cases of acute myocardial infarction (MI) and 214 population-based control subjects free of clinical CHD.

Results 38.0% of cases and 30.8% of controls were *cagA* seropositive (p=0.08). In subjects <55 years old, there was a 2.25-fold (95% CI, 1.12-4.53, p=0.01) increase in MI risk associated with *cagA* seropositivity. Logistic regression analysis showed a significant interaction (p=0.03) between and the effect of *cagA* seropositivity on risk of MI with an average 30% (3-49%) decline in the odds ratio per decade. There was no significant association of *cagA* status with classical CHD risk factors and adjustment of the logistic regression for the co-variates had no significant impact on either the overall association or the interaction with age. 60.2% of cases and 53.7% of controls were *H. pylori* seropositive (odds

ratio 1.12 (0.83-1.51), p=0.43). *H. pylori* seropositivity was not increased in young cases and did not show interaction with age.

Interpretation; The association of chronic *H. pylori* infection with risk of MI appears to be restricted to *cagA* bearing strains. As with other vascular risk factors the association was age-dependent and stronger in younger individuals. Genetic heterogeneity of *H. pylori* may explain some of the discordant findings with regard to the association of *H. pylori* with CHD.

6.1 Introduction.

H. pylori is commonly acquired in childhood and is usually chronic (Graham *et al*, 1991). Elevated concentrations of IgG antibodies to *H. pylori* are a fairly reliable indicator of infection, either present or past. Recently, in conjunction with a variety of other chronic infections, an interest in the possible association between *H. pylori* infection and coronary heart disease (CHD) has developed (Danesh *et al*, 1997). Mendall and colleagues were the first group to report a higher prevalence of *H. pylori* seropositivity in patients with CHD compared with healthy volunteers (Mendall *et al*, 1994). Subsequent studies have produced conflicting findings (McDonagh *et al*, 1997; Murray *et al*, 1995; Niemela *et al*, 1996; Patel *et al*, 1995; Rathbone *et al*, 1996; Wald *et al*, 1997) and the significance of the association remains uncertain (Danesh *et al*, 1997). Confounding by the strong relationship of *H. pylori* infection to other CHD risk factors such as age and social class may, at least partly, explain the contradictory results. However, there is increasing evidence that inflammation plays an important role in CHD (Entman *et al*, 1993; Ross, 1993). Strains bearing the *cagA* gene provoke a heightened inflammatory response *in vivo* (Peek *et al*, 1995), and thus it is feasible that any impact of *H. pylori* infection on CHD is dependent on the type of infecting strain.

In a previous study carried out in Leicester, (Rathbone *et al*, 1996), comparing subjects with acute myocardial infarction (MI), with population-based controls, no overall association between *H. pylori* seropositivity and MI risk was observed, despite adjustment for co-variates. The blood samples from the cohorts of this study (Rathbone *et al*, 1996) were analysed for anti-CagA antibodies, as an indicator of infection with more virulent *H. pylori* strains.

6.1.1 Aims.

The aim of this work was to investigate whether the type of *H. pylori* strain is of relevance in the risk of MI in an infected individual.

6.2 Methods and Materials

6.2.1 Subjects

The recruitment of patients and controls has previously been described (Rathbone *et al*, 1996). Briefly, patients who satisfied the World Health Organisation criteria for MI (WHO *et al*, 1979) were recruited from admissions to the coronary care unit (CCU) at Leicester Royal Infirmary. The CCU, serving a population of around 300,000, accounts for >65% of admissions of cases with MI in Leicester. The period of recruitment was between July 1993 and April 1994, and > 95% of eligible subjects were recruited. Control subjects were recruited randomly from adult visitors to patients with non-cardiovascular illnesses on general medical and surgical wards at the Leicester Royal Infirmary to provide subjects likely to be representative of the source population from which the cases came.

Cases and controls filled in a standard questionnaire about their personal histories, had height and weight measured and provided blood samples for laboratory analysis. The study was approved by the local research ethics committee.

6.2.2 Biochemical measurements.

Serum total and HDL-cholesterol were measured using a Kodak Ektachem E700 CXR Automatic Analyser in a quality-controlled hospital biochemistry laboratory. For cases, the first blood sample taken after admission was used for the analysis

6.2.3 Determination of *H. pylori* and *cagA* serological status

H. pylori status was determined serologically, as described previously (Rathbone *et al*, 1996), using an established IgG ELISA assay based on an ultracentrifuged sonicate antigen. IgG antibodies to the CagA protein were quantified on whole blood samples using a commercial ELISA kit (Helicobacter p120 (CAGA) ELISA, Viva Diagnostics, Hurth, Germany). The ELISA was validated by concurrent Western Blot analysis of 207 of the samples to confirm the presence or absence of anti-CagA antibodies, using a reference strain (NCTC 11637, National Collection of Type Cultures, London, UK) as antigen. An ELISA CagA unit value of 5.2 gave the optimal sensitivity and specificity for the assay (86.7% and 85% respectively).

6.2.4 Statistical Analysis

Controls reporting a history of MI or angina were excluded from the statistical analysis.

Distribution of anti-CagA seropositivity and qualitative risk factors between cases and controls or qualitative risk factors between anti-CagA positive and negative cases or controls were compared using the chi-squared test. Quantitative sample means were compared by analysis of variance. Logistic regression was used to analyse the effects of anti-CagA seropositivity on MI status and its interaction with linear age effect adjusted for gender. Adjustment was then made for other co-variates by including them in the regression. The effects of these adjustments were tested by comparing the odds ratios associated with CagA and the corresponding age interaction term, when estimated with and without adjustment (Mickey *et al*, 1989). In these analyses, age was adjusted for by pooling estimates made at different ages to obtain Mantel-Haenszel estimates and separately, by including a linear age

term but no interaction, in a logistic regression. The changes in the odds ratio associated with anti-CagA seropositivity adjusted for age were then plotted against the appropriate age cut-off.

6.3 Results.

6.3.1 Patient details.

A total of 578 subjects' serum was analysed in terms of *H. pylori* status and the presence or absence of antibodies against the CagA protein. 22 control patients had a past history of IHD and so were excluded from the analysis of CagA as a risk factor for MI in cases versus controls.

A total of 556 subjects were analysed. 342 of these subjects were cases and 214 controls. The subjects' characteristics are summarised in Table 6.1. The data presented are the mean ± standard deviation (SD) or percent of group. Overall, the cases were significantly older than the controls and the classical risk factors associated with MI were more prevalent in cases (hypertension, diabetes, smoking, and a positive family history). Body mass index (BMI), and total and high-density lipoprotein (HDL)-cholesterol levels were similar between cases and controls.

Table 6.1 - Characteristics of cases and controls.

Characteristics	Cases (n=342)	Controls (n=214)
Age (yrs)*	65.1±11.7	54.8±12.6
Males	66.9	58.8
White	88.0	92.9
Hypertension*	33.1	16.8
Diabetes*	12.1	3.7
Current smokers*	32.5	18.2
Angina	28.3	-
Previous MI	19.3	-
+ve FH*	38.2	25.1
BMI (kg/m^2)	25.7±4.2	25.4±3.7
Total cholesterol (mmol/l)	5.6±1.2	5.6±1.0
HDL cholesterol	1.2±0.3	1.2±0.3

* $P < 0.001$ cases versus controls.

6.3.2 *H. pylori* status.

All the 578 patients were analysed in terms of their *H. pylori* status, and the data is presented in Table 6.2. Two hundred and six of the 342 cases (60.2%) and 132/236 (55.9%) controls were *H. pylori* seropositive. With the 22 control patients removed from the data, 115/214 (53.7%) controls were *H. pylori* seropositive.

Table 6.2 - *H. pylori* status in cases and controls.

	Antibodies to *H. pylori*		
	H. pylori positive	*H. pylori* negative	Total
Cases	206	136	342
Controls	132	104	236
Total	338	240	578

6.3.3 Determination of anti-CagA antibody status.

6.3.3.1 Quantitation and variability of ELISA assay.

A total of 7 ELISA assays were needed to run the 578 samples. Absorbency was measured at A_{450} and Table 6.3 shows the absorbency values across all 7 kits. Positive and negative control sera, blanks and one positive reference whole blood sample were included in each assay. This allowed day-to-day variations in the assays to be assessed and as demonstrated in Table 6.3, there was no significant discrepancies between the values on a day to day basis. The whole blood sample used was from a Registrar colleague, who had anti-CagA antibodies as determined by western Blot. 50ml of whole blood was frozen in small aliquots and then used in each run.

Table 6.3- Absorbency values across all 7 kits.

ABSORBENCY VALUES AT A_{450}			
Calibrator	Positive control	Negative control	Blood control
1.128	1.436	0.051	1.361
1.236	1.531	0.049	1.666
1.139	1.484	0.047	1.421
1.161	1.570	0.046	1.622
1.098	1.403	0.052	1.564
1.132	1.483	0.038	1.556
1.141	1.615	0.030	1.543
1.147 (±0.04)	1.503 (±0.069)	0.045 (±0.007)	1.533 (±0.100)

6.3.3.2 Calculation of unit values and determination of anti-CagA antibody status.

The unit value of the samples were calculated using the equation:

(sample absorbency: calibrator absorbency) x unit value of calibrator = unit value of sample

The ELISA unit values for the positive, negative and blood controls across all 7 kits are detailed in Table 6.4

Table 6.4- ELISA unit values across all 7 kits.

ELISA unit values			
Calibrator	Positive control	Negative control	Blood control
23U	29.2U	1.0U	28.9U
23U	28.5U	0.9U	31.0U
23U	30.0U	0.9U	28.7U
23U	31.1U	0.9U	32.1U
23U	29.4U	1.1U	32.8U
23U	30.1U	0.8U	31.6U
23U	32.6U	0.6U	31.1U
23U	30.1U (±1.3U)	0.9U (±0.1U)	30.9U (±1.4U)

The unit value of the sample defined whether a patient was positive or negative using the cut-off values suggested in the manufacturer's instructions:

<5	negative
5-7.5	borderline
7.5-25	positive
>25	strong positive

Samples with a unit value of 5-7.5 were considered equivocal and assigned to positive or negative by Western Blotting. Equivocal samples and their immunoblotting data are presented in Table 6.5.

Table 6.5 - Equivocal samples with unit values in the range 5-7.5 units

Patient ID	ELISA VALUE	Anti-CagA Ab immunoblot data
2148	5.0	negative
74	5.0	negative
69	5.0	**positive**
2188	5.1	negative
2101	5.1	negative
2009	5.1	negative
1378	5.1	negative
523	5.1	**positive**
2161	5.2	**negative**
362	5.2	**negative**
2143	5.3	**negative**
2102	5.3	positive
2006	5.3	**negative**
1271	5.3	positive
494	5.3	**negative**
383	5.3	positive
2137	5.4	positive
2136	5.4	positive
473	5.4	positive
2160	5.5	positive
260	5.5	**negative**
112	5.5	**negative**
2167	5.6	**negative**
485	5.6	**negative**
391	5.6	positive
2054	5.7	**negative**
296	5.7	**negative**
20	5.7	positive

Patient ID	ELISA value	Anti-CagA Ab Immunoblot data
2185	5.8	positive
1114	5.8	positive
504	5.8	positive
2138	5.9	positive
2092	5.9	positive
2079	5.9	positive
586	5.9	**negative**
2272	6.0	positive
2179	6.0	**negative**
2055	6.0	**negative**
1373	6.0	positive
36	6.0	**negative**
96	6.2	positive
2186	6.3	**negative**
2095	6.3	positive
437	6.3	positive
269	6.3	positive
2046	6.5	positive
1050	6.5	positive
1221	6.6	positive
419	6.6	positive
411	6.7	positive
286	6.7	positive
2127	6.8	positive
535	6.8	positive
98	6.8	positive
1078	7.0	positive
2052	7.1	positive
425	7.1	positive
205	7.1	positive
9	7.2	positive
2159	7.5	positive

Red text represents false positive and negative ELISA samples, using an ELISA cut off value of 5.2U (see text ahead).

6.3.4 Calculation of the anti-CagA ELISA sensitivity and specificity values.

In order to validate the anti-CagA ELISA for use on the study population, 207 samples were randomly assigned for immunoblotting (data in Appendix). Using this data, a "cut-off" value was determined which gave the best sensitivity and specificity values for this population. A cut-off value of 5.2 U was used and the ELISA sensitivity and specificity determined:

Sensitivity

$$\frac{\text{True positives}}{\text{True positives + false negatives}}$$

Specificity

$$\frac{\text{True negatives}}{\text{True negatives + false positives}}$$

"True positives" = positive ELISA samples which are positive for anti-CagA antibodies by immunoblotting.

"True negatives"= negative ELISA samples which are negative for anti-CagA antibodies by immunoblotting.

False positives have an ELISA unit value of 5.2 or greater, but are negative for anti-CagA antibodies by immunoblotting.

False negatives have an ELISA unit value of less than 5.2, but are positive for anti-CagA antibodies by immunoblotting.

Excluding the equivocal samples, there were 14 false negatives and one false positive sample. Table 6.6 summarises the data on the false positive and negative samples. Including the

equivocal samples with a cut off of 5.2 U, a further 2 samples were false negatives and 16 were false positive (Table 6.5, red text).

Table 6.6 - False positive and false negative samples of the anti-CagA ELISA.

Patient ID	ELISA value	Anti-CagA antibodies on immunoblot
31	4.5	positive
261	4.1	positive
189	3.3	positive
26	3.0	positive
389	2.9	positive
173	2.5	positive
354	2.5	positive
13	2.2	positive
372	2.1	positive
385	2.1	positive
85	1.9	positive
395	1.8	positive
320	1.6	positive
303	1.2	positive
Total false -ve's		14
363	8.7	negative

Using a cut off value of 5.2 the ELISA sensitivity and specificity for this study population were:

1. **Sensitivity**:

$$\frac{111}{111 + 16} \times 100 = \mathbf{87.4\%}$$

2. **Specificity**:

$$\frac{96}{96 + 17} \times 100 = \mathbf{85\%}$$

6.3.5 *cagA* status and MI risk.

A total of 204/578 subjects were anti-CagA seropositive. The distribution of anti-CagA seropositivity in cases and controls is shown in Table 6.7.

Table 6.7- Anti-CagA for all ages, in 578 patient samples (based on ELISA and western blot results).

Antibodies to CagA			
	CagA positive	CagA negative	Total
Cases	130	212	342
Controls	74	162	236
Total	204	374	578

Table 6.8 summarises the distribution of risk factors in cases according to CagA status. The data presented are the mean ± SD or percent of the group. There was no significant association between anti-CagA seropositivity with any of the risk factors and adjustment of the logistic regression for the co-variates had no significant impact on either the overall association (p=0.85) or the interaction with age (p=0.81).

Table 6.8 Distribution of risk factors in cases according to CagA status.

Risk factors	CagA+ (n=130)	CagA- (n=212)	p value
Age (yrs)	64.8±11.9	65.2±11.7	0.77
Males	65.4	67.9	0.63
White	85.4	89.6	0.24
Hypertension	37.2	30.7	0.23
Diabetes	10.7	12.9	0.54
Current smokers	30.9	33.5	0.56
Angina	28.9	28.0	0.85
Previous MI	19.7	19.0	0.89
+ve FH	37.5	38.6	0.88
BMI (kg/m^2)	25.8±4.0	25.6±4.3	0.71
Total cholesterol (mmol/l)	5.7±1.3	5.6±1.2	0.43
HDL cholesterol	1.25±0.35	1.20±0.34	0.34

Data presented are mean ± SD or percent of group. FH= family history in first degree relative; BMI= body mass index; HDL= high-density lipoprotein.

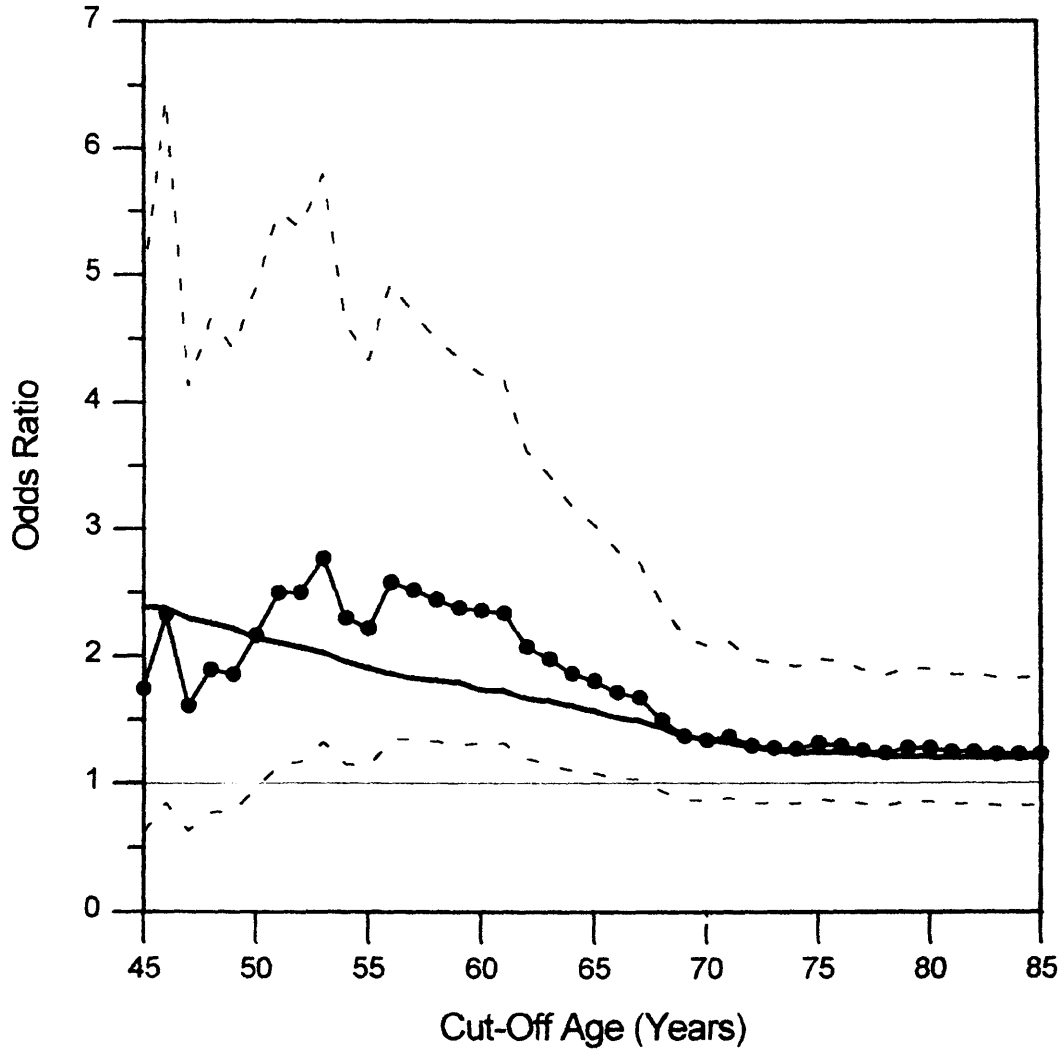
The data was statistically analysed after the exclusion of the control patients with a past history of IHD, leaving a study population of 556, 196 of whom had anti-CagA antibodies (22 controls with a past history of IHD). Because of the significant difference in age distribution of cases and controls, the potential impact of age on the association of anti-CagA seropositivity with the risk of MI was examined. Table 6.9 shows the distribution of anti-CagA seropositivity in all subjects (556), patients under 65 and patients under the age of 55. When the group was analysed as a whole there was no significant association: 38% of cases and 30.8% of controls were anti-CagA seropositive (p=0.08). In subjects under 65 years of age (n=153 cases, 153 controls) there was a 1.80-fold (p=0.02) increase in MI risk associated with CagA seropositivity, which increased further to 2.25-fold (p=0.01) in patients under 55 years old.

Table 6.9 Distribution of anti-CagA seropositivity in cases and controls.

Subjects	Whole group		<65 years		< 55 years	
	CagA +ve	CagA –ve	CagA+ve	CagA-ve	CagA +ve	CagA -ve
Cases	130	212	57	96	27	39
Controls	66	148	38	115	28	91
	OR 1.38 (0.94 – 2.01) P = 0.08		OR 1.80 (1.07-3.03) P=0.02		OR 2.25 (1.12 – 4.53) P = 0.01	

Figure 6.1 shows the statistical relationship between age, anti-CagA seropositivity and risk of MI. In the Mantel-Haenzel analysis, the age and gender adjusted odds ratio remained significant to an age of about 65 years. A logistic regression analysis showed a significant interaction ($p=0.03$) between age and the effect of anti-CagA seropositivity on risk of MI. The odds ratio associated with anti-CagA seropositivity declined on average by 30% per decade (95% CI, 3-49%) (Figure 6.1 solid line). A similar analysis for *H. pylori* seropositivity showed no similar increase in younger subjects or interaction with age (Rathbone et al, 1996).

Figure 6.1



6.3.6 Anti-CagA antibodies and *H. pylori* status.

Table 6.10 shows the distribution of anti-CagA seropositivity in *H. pylori* negative and positive subjects. In 30 of the subjects, there was discrepancy between the *H. pylori* status as defined by the anti-*H. pylori* ELISA and the anti-CagA ELISA. Details of these 30 patients are presented in Table 6.11. All 30 patients were *H. pylori* seronegative on ELISA (Rathbone *et al*, 1996), but had detectable antibodies to CagA on the anti-CagA ELISA. Immunoblot data was available on 23/30 subjects, 13 (43%) of which had anti-CagA antibodies as defined by immunoblot.

Table 6.10 - Anti-CagA antibodies and *H. pylori* status in cases and controls

H. pylori status	Antibodies to CagA.		
	Positive	Negative	Total
H. pylori positive patients			
Cases	109	97	206
Controls	65	67	132
H. pylori negative patients			
Cases	21	115	136
Controls	9	95	104
Total	204	374	578

Red text = 30 discrepancies with anti-CagA activity in *H. pylori* negative patients.

Table 6.11 - *H. pylori* negative patients who have anti-CagA antibodies as defined by anti-CagA ELISA (where 5.2U is the cut off value).

Patient ID	*H. pylori* ELISA	Anti-CagA ELISA	Immunoblot
14	negative	9.4	**positive**
36	negative	6.0	**positive**
113	negative	14.2	negative
165	negative	10.5	**positive**
197	negative	17.4	**positive**
209	negative	10.0	no data
237	negative	7.7	**positive**
260	negative	5.5	negative
324	negative	18.3	no data
362	negative	5.2	negative
383	negative	5.3	**positive**
391	negative	5.6	**positive**
411	negative	6.7	**positive**
425	negative	7.0	no data
437	negative	6.3	**positive**
473	negative	5.4	**positive**
485	negative	5.6	negative
494	negative	5.3	negative
535	negative	6.8	**positive**

Patient ID	*H. pylori* ELISA	Anti-CagA ELISA	Immunoblot
1271	negative	5.3	**positive**
1373	negative	6.0	**positive**
2030	negative	12.4	no data
2054	negative	5.7	negative
2055	negative	6.0	negative
2121	negative	13.7	no data
2143	negative	5.3	negative
2154	negative	11.4	no data
2179	negative	6.0	negative
2186	negative	6.3	negative
2277	negative	9.4	no data

Red text represents patients who are *H. pylori* negative by ELISA, but have anti-CagA antibodies as defined by both immmunoblot and Anti-CagA ELISA

6.4 Discussion.

The finding in this study of a specific association between *cagA* positive strains of *H. pylori* and risk of premature myocardial infarction adds to the current debate about the possible role of chronic bacterial infections in the pathogenesis of coronary heart disease (Danesh *et al*, 1997; Ridker, 1998). This study provides evidence that any effect of *H. pylori* infection on CHD risk may also be related to the type of infecting strain. Such risk appeared independent of classical risk factors for CHD.

6.4.1 *cagA* positive strains and risk of MI.

The specific association of *cagA* positive strains with MI risk is biologically plausible. *cagA* and the associated vacuolating cytotoxin (VacA), define type I strains (Xiang *et al*, 1995) which produce a more marked gastric inflammatory response, including the induction of IL-6, IL- 8, and the tumour necrosis factor alpha, compared with type II (*cagA* negative) strains (Peek *et al*, 1995; Yamaoka *et al*, 1997). Type I strains are associated with PUD (Atherton *et al*, 1995) and gastric cancer (Blaser *et al*, 1995). This enhanced response in type I strains is thought to be due to the presence of a 40 Kb DNA insertion the '*cag* pathogenicity island', which encodes for several virulent factors which promote the inflammatory response (Censini *et al*, 1996). Increasing evidence suggests that inflammation (both systemically as well as locally) plays an important role in the development of CHD and particularly the progression to acute coronary syndromes (Entman *et al*, 1993; Ridker, 1998; Ross, 1993). Systemic markers of inflammation such as C-reactive protein and acute-phase reactants such as fibrinogen and serum amyloid A have been shown to be prospectively associated with MI risk (Liuzzo *et al*,

1994; Kuller *et al*, 1996), and part of the beneficial effects of aspirin on coronary risk may be related to its anti-inflammatory rather than anti-platelet effect (Ridker *et al*, 1997). Thus, it is possible that *cagA* positive strains increase the risk of MI through the promotion of an enhanced inflammatory response. *H. pylori* seropositivity has been associated with elevated levels of fibrinogen and C-reactive protein (Mendall *et al*, 1996; Patel *et al*, 1994), although, as in the case of the association with CHD, results have been mixed and a recent meta-analysis suggested that the correlations were due to chance or publication bias (Danesh *et al*, 1998). However, studies in such populations have not taken into account the genetic heterogeneity of *H. pylori.*

The findings of this study support the recent report of Pasceri and colleagues (Pasceri *et al*, 1998), published whilst this study was independently under way. In 88 patients with ischaemic heart disease (age, 57±8 years, 74 men) and in 88 age- and sex-matched controls, they observed a 3.8-fold adjusted increase in risk of IHD in *cagA* positive subjects (prevalence of CagA seropositivity; cases 43%, controls 17%). Interestingly, they also observed an increase in overall prevalence of *H. pylori* seropositivity in cases (62% versus 40%). Prevalence of *cagA* negative strains was similar in cases and controls (19% versus 23%). The study of Pasceri and colleagues (Pasceri *et al*, 1998) included patients with a range of coronary syndromes including severe unstable angina, acute MI and chronic stable angina, while this larger study focused on acute MI only. Although the prevalence of infection by *cagA* positive strains was similar in their three patient groups, further studies are needed to define the precise relationship of infection with *cagA* bearing strains with different coronary syndromes. Despite this, the findings of this study, in conjunction with Pasceri's and colleagues, emphasise the

point that the ability to detect any association of *H. pylori* seropositivity with CHD may depend not only on the overall population prevalence of *H. pylori* infection, but also on the relative proportion infected with *cagA* positive strains. There is a wide variation in CagA seroprevalence in different countries (Perez-Perez *et al*, 1997).

6.4.2 Age, risk of MI and CagA seropositivity

The finding that the association of risk of MI with *cagA* positive *H. pylori* strains was age-dependent is not entirely unexpected as the associations of most vascular risk factors with CHD tend to be stronger in the younger than older individuals. Although this is the most likely explanation, it needs to be noted that several studies have shown that the accuracy of serological testing for *H. pylori*, and in particular specificity, declines with age, (Schembri *et al*, 1993; Stevens *et al*, 1997). This decline in accuracy is most marked in those over the age of 45. The reasons for this are unclear but cross-reactivity between antibodies due to increased antigenic exposure with age may be a factor.

6.4.3 Anti-CagA antibodies in *H. pylori* seronegative patients

Thirty patients in this study were *H. pylori* seronegative on ELISA, but had detectable antibodies to CagA on the anti-CagA ELISA. Immunoblot data confirmed the presence of anti-CagA antibodies in 13 (43%). Several groups have also noted CagA reactivity in *H. pylori* seronegative individuals (Fusconi *et al*, 1997; Parsonnet *et al*, 1997). In fact, one study looking at anti-CagA reactivity in *H. pylori* negative subjects, concluded that the 10% CagA positivity in *H. pylori* seronegative patients was not a false positive result and that these patients should be managed as clinically *H. pylori* positive (Fusconi *et al*, 1997). However, the reasons for this discrepancy remain uncertain. Possible explanations are the persistence of antibodies to CagA after the elimination of other anti-*H. pylori* antibodies by the immune system, or/ and cross reactivity of antibodies resulting in false positive results.

6.4.4 Criticisms and considerations for future studies

This study has several limitations common to cross-sectional studies. The possibility of unrecognised population stratification for relevant factors influencing the findings cannot be excluded. Specifically, socio-economic status is known to influence both the prevalence of *H. pylori* infection and risk of CHD (Danesh *et al*, 1997; Ridker, 1998). Although the control group were recruited form hospital visitors, specifically to provide healthy subjects likely to be representative of the source population from which the cases came, details of socio-economic status were not obtained and so could not be adjusted for. In addition, although the effect of infection with *cagA* positive strains appeared independent of classical CHD risk factors, our study does not provide mechanistic information.

The association of *cagA* strains of *H. pylori* on risk of CHD needs to be confirmed in further, ideally prospective, studies and whether the effect is mediated through an enhanced inflammatory response needs to be determined. Such studies are important because they may not only provide important insights on the pathophysiological basis of coronary syndromes, but also because of the potential, if the association is proven, of new forms of therapeutic and preventative treatments directed towards infection eradication.

6.4.5 Summary and significance of the findings

This work shows a significant and age-dependent association of infection with *cagA* positive strains of *H. pylori* and risk of MI. These findings suggest that genetic heterogeneity of *H. pylori* may explain some of the discordant findings with regard to the association of *H. pylori* with CHD.

Chapter 7

General Discussion

7.1 Overview of original objectives

7.1.1 Main aims.

H. pylori infection is common and remains largely asymptomatic in the majority of individuals. However, *H. pylori* associated pathology can cause serious morbidity. The identification of risk factors associated with disease has focused on both host and bacterial factors. The clinical importance of *H. pylori* strain type in the pathogenesis of gastroduodenal pathology is poorly understood, but two bacterial virulence determinants have been identified as potentially important; *vacA* and *cagA* and their gene products, the vacuolating cytotoxin and CagA. The previous work looking at these genes, the cytotoxin and CagA in relation to disease has been previously predominantly based *in vitro* with typing of cultured isolates and on a non-UK study population.

This thesis set out to assess if it was possible to type *vacA* and *cagA* directly from biopsy DNA without the need for culture, and to assess if *in vitro* strain types are representative of *in vivo* strain types. The work aimed to establish the clinical relevance of *cagA* and *vacA* subtypes in relation to macroscopic and microscopic gastroduodenal pathology in a dyspeptic UK population, and the association of CagA size variability in relation to disease. In addition, the thesis sought to address the use of serological detection of anti-CagA antibodies and anti-VacA antibodies as a means of diagnosing *H. pylori* infection.

The possible influence of *H. pylori* virulence determinants on the association of *H. pylori* and ischaemic heart disease may account for some of the discrepancies between studies. This work intended to establish if *cagA* influenced the relationship of *H. pylori* with CHD.

7.2 Principal findings.

7.2.1 *vacA, cagA* and disease.

The initial work looking at *cagA* and *vacA* alleles was carried out on cultures isolates, and showed an association between *cagA* and *vacA* s1 and m1 with PUD (Atherton *et al*, 1995). The work detailed in this thesis, based on a dyspeptic Leicester population, demonstrates that it is possible to directly type *cagA* status and the *vacA* s and m alleles using PCR amplification, without the need for culture. The results highlight that *in vitro* strain types are not always representative of *in vivo* strain types, with a 20% discrepancy rate, particularly in the presence of mixed infections. It is increasingly being demonstrated that mixed infections are common within an individual, thus supporting the future use of direct DNA PCR as a typing method as opposed to relying on cultured isolates.

The original identification of *cagA* and *vacA* lead to a hope of identifying individuals at risk of developing serious sequel from infection with *H. pylori*. However, further work has shown that the relationship between these virulence factors and gastroduodenal pathology is not as straightforward as initially thought, and this is highlighted by the findings in this study. A wide geographical difference between the *cagA* positivity rates and pathology is increasingly being demonstrated, particularly in Japan and China where strains with *cagA* are common,

both in patients with and without pathology (Maeda *et al*, 1997; Pan *et al*, 1997; Shimoyama *et al*, 97). Disappointingly, the use of *cagA* as a marker of patients at risk of developing *H. pylori* related pathology has similarly been demonstrated to be unreliable in this study, and is part of an ever increasing body of evidence that *cagA* is not a useful discriminator of strain virulence on a world-wide basis. However, *cagA* does seem to be important at a cellular level, with *cagA* acting as a marker for the *cag* pathogenicity island, which encodes several genes involved in inducing pro-inflammatory cytokines (Tummuru *et al*, 1996). The current work demonstrated a significant association between *cagA*, an increased antral inflammatory response, and density of bacterial colonisation which is in keeping with this hypothesis.

The association of *vacA* s1 with PUD has likewise been shown to vary considerably between different populations (Ito *et al*, 1997; Maeda *et al*, 1998) and the findings of this study are supportive of this, with 83% of NUD strains being of the type s1. Further still, various groups have demonstrated further heterogeneity in the *vacA* mid region (Stephens *et al*, 1998; Strobel *et al*, 1998; Wang *et al*, 1998) and a new s1c type of *vacA* has been identified amongst strains in East Asia (van Doorn *et al*, 1998). In 6 of our strains, the mid regions were non-typeable suggesting greater heterogeneity. With the identification of new *vacA* s subtypes, and such geographical variation in the association of *vacA* type and disease, the role of this potential virulence determinant becomes ever less certain, and it remains to be seen if the *vacA* s and m types are clinically relevant. There is also uncertainty regarding *in vitro* vacuolating cytotoxin activity, with work showing that the m2 toxin does in fact have vacuolating activity when added to primary cultured human gastric cells and acts on HeLa cell lines when expressed intracellularly (Pagliaccia *et al*, 1998).

7.2.2 CagA size, anti-CagA and anti-VacA antibodies and disease.

The importance size variation of the CagA protein is uncertain, but several studies have shown that proteins with a higher molecular weight are associated with gastric cancer (Sepulveda *et al*, 1997; Yamaoka *et al*, 1998). There was no association between molecular weight size of the CagA proteins, PUD, bacterial density or antral inflammation in this study, but there was an association of higher molecular weight sizes with gastric cancer. This, along with the findings of others may be of some relevance in the pathogenesis of *H pylori* related gastric cancer. It would be interesting to look at the size of CagA in relation to precursors of gastric carcinogenesis such as intestinal metaplasia and gastric atrophy and even to look at a possible association with MALT lymphomas.

In vitro studies demonstrate an association between cytotoxin activity and the genotype *vacA* s1/m1 (Atherton *et al*, 1995). We did not have the facilities or expertise to study directly *in vitro* cytotoxin activity in our strains, but the presence of anti-VacA antibodies can act as a measure of *in vivo* detection of the vacuolating cytotoxin. Using this principle, our results did not show a correlation between the presence of anti-VacA antibodies and *in vivo vacA* s1 or m1 type, although all three s2 strains had no detectable anti-VacA antibodies. One explanation for this result and discrepancy with *in vitro* studies is that, as highlighted by Pagliaccia and colleagues, that *in vitro* studies of cytotoxin activity is dependent on the cell line used and not necessarily representative of *in vivo* activity (Pagliaccia *et al*, 1998). In addition, cytotoxin activity does not directly correlate with cytotoxin production. *In vivo* detection of *cagA* correlated with detection of anti-CagA antibodies, but there was an 8% discrepancy. Most importantly, this work demonstrates that the use of serological methods as a diagnostic means

of detecting *H. pylori* infection should be used with caution. This is particularly important in a society where serological testing for *H. pylori* is becoming increasingly common, particularly in the General Practice, as a means of diagnosing *H. pylori* pathology in symptomatic patients. and validated for use on the local population first.

7.2.3 *cagA* and ischaemic heart disease

Only one small study, carried out in Italy, has looked at the association of *cagA* with CHD (Pasceri *et al*, 1998); an association between a variety of acute coronary syndromes, *H. pylori* and anti-CagA antibodies was shown. The current work demonstrated an age-dependent association between anti-CagA antibodies and risk of MI, but no association with *H. pylori* infection as a whole. These findings may help to account for the discrepancies found between different study groups looking at the association between *H. pylori* and CHD, and have potentially important clinical implications. Further prospective work, with a suitable control group, is necessary to establish if a true causal association exists, and therefore influence management of CHD.

7.3 Shortcomings and future considerations.

Limitations and criticisms of this work have been discussed in relation to each chapter, but some important points are highlighted below.

One particular area of further work which would be particularly useful is data on the *in vitro* vacuolating cytotoxin activity of the Leicester bacterial isolates. This data would enable

further comparisons to be made with the work of Atherton and colleagues, and that of others, in particular the work based in Japan, Taiwan and China. In addition, associations between *in vivo* strain types, *in vitro* cytotoxin activity and serological detection of anti-VacA and anti-CagA antibodies could be analysed. Southern hybridisation of the PCR products is an additional technique which could be used to improve sensitivity of detection of the PCR products, particularly where there were discrepancies between the gastric sites. Further genetic analysis of the hypervariable regions at the 3' end of the *cagA* gene may have provided interesting data for examining the relationship between CagA size variability and gastroduodenal pathology.

The number of *vacA* s2 strains identified in this study was small, limiting analysis of s2 types with macroscopic and microscopic disease and comparison with s1 types. Analysis of more strains would possibly yield more s2 types enabling further statistically significant conclusions to be drawn. However, as concluded from the results, s2 may actually be a marker of the absence of disease, and therefore studying asymptomatic patients may yield interesting results. The number of cancer patients was small. Positive associations have been demonstrated between gastric cancer, *cagA*, detection of anti-CagA antibodies and higher molecular weight sizes of CagA, but the small number of patients limited the power calculations. Additional cases studied would help confirm the associations, and it would also be interesting to examine the *cagA* status in relation to fundal as well as antral carcinomas.

Like all the studies looking at the association of coronary heart disease with *H. pylori*, a comparative control group is imperative. Although this work looking at anti-CagA antibodies

and MI used controls likely to be representative of the source population from which the cases came, socioeconomic data was not collected. In addition, measurements of inflammatory markers such as CRP and fibrinogen would help to establish if an inflammatory mechanism is responsible for the observed association. Further prospective studies of large populations are needed to look at the role of *cagA* and CHD.

7.4 Summary

It is possible to directly type *cagA* and *vacA* s and m regions from biopsy DNA, and may in fact be the preferred typing technique. This work, in conjunction with the recent published work of other groups, demonstrates that the relationship between gastroduodenal pathology, *cagA* and *vacA* s and m types is not as clear-cut as initially hoped. Neither *cagA* nor specific *vacA* subtypes can be used as reliable clinical markers for screening dyspeptic populations and targeting treatment, and there are concerns with relying on *in vitro* studies. An increasing market for serological detection of virulent determinants such as CagA is a tempting method of screening for the clinician, but the evidence presented in this thesis does not support the use of such methods.

The significant association of *cagA* positive strains with CHD is an important finding, with potential important implications for both the clinical management of CHD in infected individuals, and in the role of inflammation in the pathogenesis of atherosclerosis.

A1 APPENDIX I
Buffers, Reagents and Solutions

A1.1 Polymerase Chain Reaction and DNA preparation.

A1.1.1 10 x PCR Buffer.

Tris - HCl (pH 8.8)	100mM
KCl	500mM
$MgCl_2$	15mM
Triton x 100	1% (v/v)

A1.1.2 50 x TAE Buffer (for 500 ml).

Tris base	121g
Glacial acetic acid	28.55ml
0.5M EDTA (pH 8.0)	50ml

A1.1.3 Digestion Buffer (pH 8.0).

NaCl	100mM
Tris-HCl	10mM
EDTA	1mM

A1.1.4 DNA Loading buffer (per 10 ml).

50 x TAE buffer	1.2ml
Glycerol	4ml
Bromophenol Blue	0.025g
Nanopure water	4.8ml

A1.1.5 Phosphate Buffered Saline (pH 7.5)

Na_2HPO_4 (Disodium hydrogen orthophosphate)	80mM
NaH_2PO_4 (Sodium dihydrogen orthophosphate)	20mM
NaCl (Sodium chloride)	100mM

Distilled water is added to make up the volume.

A.1.1.6 Constituents of the 2`-Deoxynucleoside 5`-Triphosphate (dNTP) solution.

10mM each of dATP , dTTP , dCTP , dGTP , are mixed and diluted in 10mM Tris buffer (pH 8.0)

The 2`-deoxynucleoside 5`-triphosphates are supplied by Pharmacia Biotech .

A1.1.7 PCR Reaction Mix.

PCR reaction mix of 50µl was constituted using the following substrates :

10 x PCR Buffer	5.0 µl
dNTP mix	1.0 µl
primer 1	0.5 µl
primer 2	0.5 µl
PCR water	42 µl
Taq Polymerase(Biotaq^{TM}DNA polymerase)	0.4 µl (0.4µl equals 2 units)

A1.2 Western Blotting.

A1.2.1 SDS-PAGE Loading Buffer (3x).

dH_2O	0.2 ml
0.5M Tris-HCl (pH 6.8)	1.8ml
10% (w/v) SDS	2.4ml
2-β-mercaptoethanol	0.8ml
0.05% bromophenol blue	1.0ml
Glycerol	1.6ml

A1.2.2 7.5% Separating Gel (0.375M Tris pH 8.8).

dH_2O	4.6ml
1.5M Tris-HCL (pH 8.8)	2.5ml
10% (w/v) SDS	0.1ml
30% acrylamide	2.5ml
10% (w/v) APS	0.1ml
TEMED	0.004ml

A1.2.3 4% Stacking Gel (0.125M Tris pH 6.8)

dH_2O	6.1ml
0.5M Tris-HCl (pH 6.8)	2.5ml
10% (w/v) SDS	0.1ml
30% acrylamide	1.3ml
10% (w/v) APS	0.05ml
TEMED	0.01ml

Premade acrylamide; 30% Acrylamide/BIS solution, 29:1, [Acrylamide: N,N'-Methylenbisacrylamide] (Biorad).

A1.2.4 Laemmli Electrode Running Buffer, pH 8.3 (5x)

Tris base	15g/l
glycine	75g/l
SDS	5g.l

Diluted to 1x with dH_2O for running gels.

A1.2.5 Transfer Buffer (pH 8.3).

192 mM glycine

25mM Tris

20% (v/v) methanol

0.001% (w/v) SDS

A 1.3 Materials for the Helicobacter p120 (CAGA) ELISA.

Antigen Coated Microwell Strips (p120 Antigen of *H.pylori*)	12
Wash buffer Concentrate	25ml
Sample diluent concentrate, containing 0.01% thimerosal.	30ml
Substrate solution TMB	6ml
Substrate dilution buffer	6ml
Stop solution	15ml
Anti-human IgG peroxidase conjugate concentrate, containing 0.01% thimerosal	300µl
Negative control serum, containing 0.01% thimerosal	1.5ml
IgG calibrator, containing 0.01% thimerosal	1.5ml
IgG positive control serum, containing 0.01% thimerosal	1.5ml

A1.4 Materials for Silver staining.

A1.4.1 Fixative Enhancer Solution for two 8 x 10cm mini gels.

Methanol, 50% v/v	200ml
Acetic acid, 10% v/v	40ml
Fixative enhancer, 10% v/v	40ml
Deionised distilled water, 30% v/v	120ml
Total,	400ml

A1.4.2 Staining Solution.

Deionsied water	35ml
Silver complex solution	5ml
Reduction moderator solution	5ml
Image Development reagant	5ml
Development accelerator solution	50ml

A1.4.3 Development Accelerator Solution.

50g of the development accelerator reagant is added to 1000ml of deionised water, and stored at 4°C. The solution is used at room temperature.

A1.4.4 Stop Solution.

A 5% acetic acid solution is made up as the stop solution.

APPENDIX II
Commercial Suppliers

Abbreviation	Name and address.
Amersham Pharmacia Biotech UK Ltd.	Amersham Place, Little Chalfont, Buckinghamshire, HP7 9NA
Agfa Gevaert Ltd.	27 Great West Road, Beaconsfield, Buckinghamshire, HP9 1BR
Bio-Rad Laboratories Ltd.	Bio-Rad House, Maylands Avenue, Hemel Hempstead, Hertfordshire, HP2 7TD.
Bioline	16 The Edge Business Centre, Humber Road, London, NW2 6EW
Fisher Scientific UK	Bishop Meadow Road, Loughborough, Leicestershire, LE11 5RG
Denley Instruments Limited	UK Sciences Internatioal (UK) ltd. Unit S, The Ringway Centre, Edison Road, Basingstoke, Hampshire, RG21 6YH
Genelabs Diagnostics (PTE) Ltd.	85 Science Park Drive, Singapore Science Park, Singapore 0511.
Hoefer Scientific	(see Amersham Pharmacia Biotech UK Ltd)
Oxoid	Oxoid Ltd., Wade Road, Basingstoke, Hampshire, RG24 8PW
Pierce Warriner, UK, Ltd.	44 Upper Northgate Street, Chester CH1 4EF.

Perkin-Elmer Applied Biosytems	Kelvin Close, Birchwood Science Park North, Warrington, WA3 7PB
Shield Diagnostics Ltd.	The Technology Park, Dundee, DD2 1SW.
Sigma	Sigma-Aldrich Chemical Co. Ltd., Fancy Road, Poole, Dorset, BH12 OEB
Viva Diagnostics	Breite StraBe26 D-50354, Hurth, Germany.

APPENDIX III
Abbreviations and Acronyms

ATP	Adenine Tri-phosphate
AI	acute inflammation
BMI	body mass index
BSA	bovine serum albumin
BSG	British Society of Gastroenterology
CAG	chronic active gastritis
CagA	cytotoxin associated protein
cagA	cytotoxin associated gene
CCU	Coronary Care Unit
CHD	coronary heart disease
CI	chronic inflammation
CLO	*Campylobacter* like organism
CO_2	carbon dioxide
DAB	3-3 'diaminobenzidine
dATP	2'-Deoxyadenosine 5'-Triphophate
dCTP	2'-Deoxycytidine 5'-Triphophate
dGTP	2'-Deoxyguanosine 5'-Triphophate
dTTP	2'-Deoxythymidine 5'-Triphophate
DNA	Deoxyribonucleic acid
DU	duodenal ulcer
E. coli	*Escherichia coli*
EDTA	disodium ethylene dlaminetetra-acetic acid
ELISA	Enzyme - linked immunosorbent assay
FH	family history

g	centrifugal force
G+C	guanine+cytosine
GU	gastric ulcer
GI	Gastointestinal
GCa	Gastric cancer
h	hours
HCl	hydrochloric acid
HDL	high density lipoprotein
H_2O	water
H_2O_2	hydrogen peroxide
H. acinonyx	*Helicobacter acinonyx*
H. cinaedi	*Helicobacter cinaedi*
H. felis	*Helicobacter felis*
H. fennelliae	*Helicobacter fennelliae*
H. hepaticus	*Helicobacter hepaticus*
H. influenzae	*Haemophalus influenzae*
H. mustelae	*Helicobacter mustelae*
H. pylori	*Helicobacter pylori*
H&E	Haematoxylin and Eosin stain
IgG	immunoglobulin G (γ heavy chain)
IL	Interleukin
KCl	potassium chloride
LPS	lipopolysaccharide
Ltd	limited
MALT	mucosal associated lymphoid tissue
$MgCl_2$	magnesium chloride
ml	millilitres
min	minutes

MI	Myocardial infarction
MWt	molecular weight size
NUD	Non-ulcer dyspepsia
OD	Optical density
O_2	Oxygen
OR	odds ratio
PAGE	polyacrylamide gel electrophoresis.
PAI	cag pathogenicity island
PAS	Periodic acid - Schiff reagant
PBS	Phosphate Buffered saline
PBST	Phosphate Buffered saline with Tween 20
PCR	Polymerase Chain Reaction
pH	hydrogen ion concentration
PNACL	protein nucleic acid laboratory
PUD	Peptic ulcer disease
SD	standard deviation
SDS	Sodium dodecyl sulphate
TNF α	Tumour Necrosis Factor α
TAE	Tris-Acetate-EDTA
TEMED	N,N,N',N'-Tetra-methylethylene diamine
Tris	Tris-(hydromethyl)-aminomethane
TRITON x-100	t-oxtylphenoxypolethoxyethanol
Tween 20	Polyoxytheylenesorbitan monolaurate
US	universal s primers
UV	Ultra Violet
VacA	vacuolating cytotoxin
vacA	vacuolating cytotoxin gene
vacA s type	vacuolating cytotoxin gene sequence region

vacA m type	vacuolating cytotoxin gene middle region
v/v	volume/volume
w/v	weight/volume
1-D	one dimensional

APPENDIX IV

Consent Sheet.

Over the past two or three years there ahs been a lot of information in the lay press about a bacteria, called *Helicobacter pylori*, which is found in many peoples stomachs. This bacteria has been shown to be an important factor in the development of peptic ulcer disease. The Department of Gatsroenterology at the LRI has been interested in this bacteria for a number of years and has been carrying out a number of studies into it. We are currently interested in whether certain types of the bacteria are more associated with ulcers than others. We would be grateful if you would agree to us taking some extra biopsies when we examine your stomach so we can analyse them for the type of bacteria that there are and relate this to your medical condition.

In a normal endoscopy we always take biopsies routinely and this is not associated with any significant risk. If you agree to us taking extra biopies, we will take them from the duodenum and stomach which will slightly prolong your proceedure, but not significantly. There is no significant increased risk associated with the extar biopsies. All biopsies we take be analysed in the laboratory and we hope that this study will help us understand which bacteria are the most important and therefore the most impotant to get rid of.

I AGREE TO HAVING EXTRA BIOPSIES TAKEN AS OUTLINED IN THE PROJECT ABOVE. I UNDERSTAND THE INFORMATION PROVIDED.

Signed

APPENDIX V

RESULTS DATA

A5.1 Supplementary data for Chapter 3

A5.1.1 Patient, pathology and biopsy typing details

A5.1.2 Details of biopsy typing according to gastric and duodenal site

A5.2 Supplementary data for Chapter 4

A5.2.1 Bacterial density and inflammation for all patients

A5.2 2 Bacterial density and inflammation for NUD patients

A5.2.3 Bacterial density and inflammation for PUD patients

A5.3 Supplementary details for Chapter 5

A5.3.1 Complete Helicoblot 2.0 data

A5.4 Supplementary detials of 207 MI bloods

A5.1 Supplementary data for Chapter 3

A5.1.1. Patient, pathology and biopsy typing details

ID No.	Sex	Age	clo	Code	cagA (bacterial DNA)	vacA (bacterial DNA)	cagA (biopsy DNA)	US region (biopsy DNA)	m region (biopsy DNA)	endo	Serology (blot)	Anti-CagA antibody	Anti CagA ELISA
1	M	70	P	JS127	P	s1a	P	s1a	none	N	ND	ND	ND
2	F	53	P	JS128	n	s1a/m1	n	s1a	m1	N	ND	ND	ND
3	F		P	JS129	P	s1a/m2	P	s1	m2	N	ND	ND	ND
4	F	60	P	JS131	n	s1a/m2	P	s1a	m2	N	pos	no	1.8
5	M	71	P	JS132	n	s2/m2	n	s2	m2	N	weak	no	2.8
6	F	67	P	JS135	P	s1a/m1	P	s1a	m1	N	pos	yes	19.9
7	M	50	P	JS138	P	s1a/m1	P	s1a	m1	N	ND	ND	ND
8	M	34	P	JS144	P	s1a/m2	P	s1a	m2	N	pos	yes	16.5
9	M	84	P	JS146	P	s1a/m2	P	s1a	m2	N	weak	yes	3.1
10	M	57	P	JS149	P	s1a+b/m2	P	s1a	m2	N	pos	yes	8.4
11	M	77	P	JS154	P	s1a/m2	P	s1a	m2	N	pos	yes	31.1
12	F	75	P				P	s1b	m1	N	ND	ND	ND
13	F	25	P				P	s1a	m2	N	neg	no	0.1
14	M	26	n				n	s2	m2	N	neg	no	0.9
15	M	38	P				P	s1a	m1	N	pos	yes	
16	F	43	P				n	s2	m2	N	ND	ND	ND
17	M	34	P				P	s1a	m2	N	pos	yes	36.9
18	F	74	P				P	s2	m2	N	ND	ND	ND
19	F	74	P				P	s1b	m2	N	pos	yes	9.9
20	F	20	P				P	s1a	m2	N	pos	yes	30.6
21	M	36	P				P	s1a	m1	N	pos	yes	23.1
22	F	70	P				P	s1a	m2	N	pos	yes	19.2
23	M	69	P	JS130	P	s1a/m2	P	s1	m2	DU	weak	yes	12.8
24	M	56	P	JS134	P	s1a	P	s1a	none	DU	pos	yes	27.8
25	M	62	P	JS136	n	s1a/m1	P	s1a	m1 + 2	DU	pos	yes	34.0
26	F	63	P	JS137	P	s1a/m2	P	s1a	m2	DU	pos	yes	24.2
27	M	74	P	JS142	n	s1a/m2	n	s1a	m2	DU	ND	ND	ND
28	M	72	P	JS143	P	s1a	P	s1a	m1	DU	pos	yes	22.7
29	M	52	P	JS145	n	s1a/m2	n	s1a	m2	DU	pos	no	0.6
30	M	69	P	JS147	P	s1a/m2	P	s1a+b	m2	DU	pos	yes	30.8
31	M	74	P	JS148	P	s1a/m2	P	s1a	m2	DU	pos	yes	29.9
32	M	79	P	JS150	P	s1/m2	P	s1a	m2	DU	pos	yes	4.8
33	M	34	P	JS151	P	s1/m2	P	s1a	none	DU	pos	yes	13.1
34	M	87	P	JS153	n	s1a/m2	P	s1a	m2	DU	ND	ND	ND
35	F	72	P				n	s1a	m1	DU	neg	no	0.4
36	F	61	P				P	s1a	m2	DU	pos	no	0.1
37	F	38	P				P	s1a	none	DU	pos	yes	23.4
38	M	33	P				P	s1a	m2	DU	pos	yes	37.5
39	M	50	n	JS140	P	s1a/m1	P	s1a	m1	GU	pos	yes	16.5
40	M	58	P	JS141	P	s1a/m2	P	s1a	m2	GU	pos	yes	35.0
41	M	76	P	JS152	P	s1a	P	s1a	m2	GU	ND	ND	ND
42	F	72	P				n	s1a	m2	GU	ND	ND	ND
43	F	53	P				P	s1a	m1	GU	pos	yes	28.0
44	F	74	P				P	s1b	m1	GU	pos	yes	23.4
45	M	53	n				P	s1b	m1	GU	pos	yes	22.5

46	M	75	P				n	s2	m2	GU	weak	no	0.3
47	M	46	P				**P**	s1a	m1	GU	**pos**	**yes**	**29.5**
48	M	67	n	JS133	**P**	s1a/m2	**P**	s1	m2	CA	**pos**	**yes**	**32.8**
49	M	61	P	JS139	**P**	s1/m1	**P**	s1a	m1	CA	**pos**	**yes**	**16.4**
50	F	65	P				**P**	s1b	m1	CA	**pos**	**yes**	**9.2**
51	F	71	P				**P**	s1a	none	CA	**pos**	**yes**	**33.2**
52	M	84	P				**P**	s1b	m2	CA	**pos**	**yes**	**9.2**
53	m	72					p	s1a	none	N	ND	ND	
54		30	P	JS156	n	s1b/m1	P	s1b,s2	m1,m2	DU	pos	pos	
55	M	70	P				n	s1a	m2	CA	w.pos	pos	
56	F	49	n				P	s1	m1	N	pos	pos	
57	F	66	P				P	s1b	m1	DU	pos	pos	
58	M	88	P				n	s2	m2	DU	ND	ND	
59	M	45	P	JS155	P	s1a/m1	P	s1	m1	DU	pos	pos	
60	M	77	P				n	s1	m2	Du	pos	pos	
61	F	84		JS157	n		n	s1a	m2	Gu	pos	neg	
62	M	80		JS158	p		ND	ND	ND	Ca			

A5.1.2 Details of biopsy typing according to gastric and duodenal site

Patient	code	cagA	vacA m1	vacA m2	vacA s1a	vacA s1b	vacA s2	universals
ID 1	MG1	CG1	VG1	VG2	VG3	VG4	VG5	
culture +, JS127	MG2	**CG2**	VG6	VG7	**VG8**	VG9	VG10	
cagA +, s1a only	MG3	**CG3**	VG11	VG12	**VG13**	VG14	VG15	
	MG4	**CG4**	VG16	VG17	**VG18**	VG19	VG20	
	MG5	**CG5**	VG21	VG22	**VG23**	VG24	VG25	
ID 12	MG16	CG16	VG76	VG77	VG78	VG79	VG80	
	MG17	**CG17**	**VG81**	VG82	VG83	**VG84**	VG85	
	MG18	**CG18**	**VG86**	VG87	VG88	**VG89**	VG90	
	MG19	**CG19**	**VG91**	VG92	VG93	**VG94**	VG95	
	MG20	**CG20**	**VG96**	VG96	VG98	**VG99**	VG100	
ID 13	MG36	CG36	VG176	VG177	VG178	VG179	VG180	
	MG37	**CG37**	VG181	**VG182**	**VG183**	VG184	VG185	
	MG38	**CG38**	VG186	**VG187**	VG188	VG189	VG190	
	MG39	**CG39**	VG191	**VG192**	VG193	VG194	VG195	
	MG40	**CG40**	VG196	**VG197**	VG198	VG199	VG200	
ID 3	MG41	CG41	VG201	VG202	VG203	VG204	VG205	
culture +, JS129	MG42	**CG42**	VG206	**VG207**	VG208	VG209	VG210	**s1**
cagA+, s1a/m2	MG43	**CG43**	VG211	**VG212**	VG213	VG214	VG215	**s1**
	MG44	**CG44**	VG216	**VG217**	VG218	VG219	VG220	**s1**
	MG45	CG45	VG221	VG222	VG223	VG224	VG225	
ID 14	MG51	CG51	VG251	VG252	VG253	VG254	VG255	
	MG52	CG52	VG256	**VG257**	VG258	VG259	**VG260**	
	MG53	CG53	VG261	**VG262**	VG263	VG264	**VG265**	
	MG54	CG54	VG266	**VG267**	VG268	VG269	**VG270**	
	MG55	CG55	VG271	**VG272**	VG273	VG274	**VG275**	
ID 2	MG56	CG56	VG276	VG277	VG278	VG279	VG280	
culture +, JS128	MG57	CG57	**VG281**	VG282	**VG283**	VG284	VG285	**s1**
cagA-, s1a/m1	MG58	CG58	**VG286**	VG287	**VG288**	VG289	VG290	**s1**
	MG59	CG59	**VG291**	VG292	**VG293**	VG294	VG295	**s1**
	MG60	CG60	**VG296**	VG297	**VG298**	VG299	VG300	**s1**
ID 15	MG66	**CG66**	**VG326**	VG327	**VG328**	VG329	VG330	**s1**
	MG67	**CG67**	**VG331**	VG332	**VG333**	VG334	VG335	**s1**
	MG68	**CG68**	**VG336**	VG337	**VG338**	VG339	VG340	**s1**
	MG69	**CG69**	**VG341**	VG342	**VG343**	VG344	VG345	**s1**
	MG70	**CG70**	**VG346**	VG347	**VG348**	VG349	VG350	**s1**
ID 35	MG76	CG76	VG376	VG377	VG378	VG379	VG380	
	MG77	CG77	**VG381**	VG382	**VG383**	VG384	VG385	**s1**
	MG78	CG78	**VG386**	VG387	**VG388**	VG389	VG390	**s1**
	MG79	CG79	**VG391**	VG392	**VG393**	VG394	VG395	**s1**
	MG80	no sample						

	code	cagA	m1	m2	s1a	s1b	s2	us
ID 42	MG81	CG81	VG401	VG402	VG403	VG404	VG405	s1
	MG82	CG82	VG406	VG407	VG408	VG409	VG410	s1
	MG83	CG83	VG411	VG412	VG413	VG414	VG415	s1
	MG84	CG84	VG416	VG417	VG418	VG419	VG420	s1
	MG85	CG85	VG421	VG422	VG423	VG424	VG425	s1
ID 50	MG86	CG86	VG426	VG427	VG	VG	VG	
	MG87	CG87	VG431	VG432	VG433	VG434	VG435	s1
	MG88	CG88	VG436	VG437	VG438	VG439	VG440	
	MG89	CG89	VG441	VG442	VG443	VG444	VG445	s1
	MG90	CG90	VG	VG	VG	VG	VG	
ID 51	MG91	CG91	VG451	VG452	VG453	VG	VG	
	MG92	CG92	VG456	VG457	VG458	VG459	VG460	s1
	MG93	CG93	VG461	VG462	VG463	VG464	VG465	s1
	MG94	CG94	VG466	VG467	VG468	VG469	VG470	s1
	MG95	CG95	VG471	VG472	VG473	VG474	VG475	s1
ID 43	MG96	CG96	VG476	VG477	VG478	VG	VG	
	MG97	CG97	VG481	VG482	VG483	VG484	VG485	s1
	MG98	CG98	VG486	VG487	VG489	VG	VG	s1
	MG99	CG99	VG491	VG492	VG493	VG	VG	s1
	MG100	CG100	VG496	VG497	VG498	VG	VG	s1
ID 16	MG106	CG106	VG526	VG527	VG	VG	VG	
	MG107	CG107	VG531	VG532	VG	VG	VG	s2
	MG108	CG108	VG536	VG537	VG	VG	VG	s2
	MG109	CG109	VG541	VG542	VG	VG	VG	s2
	MG110	CG110	VG546	VG547	VG	VG	VG	s2
ID 17	MG111	CG111	VG551	VG552	VG553	VG	VG	
	MG112	CG112	VG556	VG557	VG558	VG559	VG560	s1
	MG113	CG113	VG561	VG562	VG563	VG	VG	s1
	MG114	CG114	VG566	VG567	VG568	VG	VG	s1
	MG115	CG115	VG571	VG572	VG573	VG	VG	s1
ID 36	MG116	CG116	VG576	VG577	VG578	VG	VG	
	MG117	CG117	VG581	VG582	VG583	VG584	VG585	s1
	MG118	CG118	VG586	VG587	VG588	VG	VG	s1
	MG119	CG119	VG591	VG592	VG593	VG	VG	s1
	MG120	CG120	VG596	VG597	VG598	VG	VG	s1
ID 23	MG121	CG121	VG601	VG602	VG603	VG	VG	
culture +, JS130	MG122	CG122	VG606	VG607	VG608	VG609	VG610	s1
cagA+, s1a/m2	MG123	CG123	VG611	VG612	VG613	VG	VG	s1
	MG124	CG124	VG616	VG617	VG618	VG	VG	s1
	MG125	CG125	VG621	VG622	VG623	VG	VG	s1

	code	cagA	m1	m2	s1a	s1b	s2	us
ID 18	MG126	CG126	VG626	VG627	VG	VG	VG	
	MG127	CG127	VG631	**VG632**	VG	VG	VG	s2
	MG128	CG128	VG636	**VG637**	VG	VG	VG	s2
	MG129	**CG129**	VG641	**VG642**	VG	VG	VG	s2
	MG130	CG130	VG646	**VG647**	VG	VG	VG	s2
ID 52	MG131	CG131	VG651	VG652	VG	VG	VG	
	MG132	**CG132**	VG656	**VG657**	VG658	**VG659**	VG660	
	MG133	**CG133**	VG661	**VG662**	VG	**VG664**	VG	s1
	MG134	**CG134**	VG666	**VG667**	VG	**VG669**	VG	s1
	MG135	**CG135**	VG671	**VG672**	VG	**VG674**	VG	s1
ID 44	MG136	CG136	VG676	VG677	VG	VG	VG	
	MG137	**CG137**	**VG681**	VG682	VG683	**VG684**	VG685	s1
	MG138	**CG138**	**VG686**	VG687	VG	**VG689**	VG	s1
	MG139	**CG139**	**VG691**	VG692	VG	**VG694**	VG	s1
	MG140	**CG140**	**VG696**	VG697	VG	**VG699**	VG	s1
ID 45	MG141	CG141	VG701	VG702	VG	VG	VG	
	MG142	**CG142**	**VG706**	VG707	VG708	**VG709**	VG710	
	MG143	**CG143**	**VG711**	VG712	VG	**VG714**	VG	s1
	MG144	**CG144**	**VG716**	VG717	VG	**VG719**	VG	s1
	MG145	**CG145**	**VG721**	VG722	VG	**VG724**	VG	s1
ID 19	MG146	CG146	VG726	VG727	VG	VG	VG	
	MG147	**CG147**	VG731	**VG732**	VG733	**VG734**	VG735	
	MG148	**CG148**	VG736	**VG737**	VG	**VG739**	VG	s1
	MG149	**CG149**	VG741	**VG742**	VG	**VG744**	VG	s1
	MG150	**CG150**	VG746	**VG747**	VG	**VG749**	VG	s1
ID 4	MG151	CG151	VG751	**VG752**	**VG753**	VG	VG	s1
culture +, JS131	MG152	**CG152**	VG756	**VG757**	**VG758**	VG759	VG760	s1
cagA-, s1a/m2	MG153	**CG153**	VG761	**VG762**	**VG763**	VG	VG	s1
	MG154	**CG154**	VG766	**VG767**	**VG768**	VG	VG	s1
	MG155	**CG155**	VG771	**VG772**	**VG773**	VG	VG	s1
ID 5	MG156	CG156	VG776	**VG777**	VG	VG	VG	s2
culture +, JS132	MG157	CG157	VG781	**VG782**	VG	VG	VG	s2
cagA-, s2/m2	MG158	CG158	VG786	**VG787**	VG788	VG789	VG790	
	MG159	CG159	VG791	**VG792**	VG	VG	VG	s2
	MG160	CG160	VG796	**VG797**	VG	VG	VG	s2
ID 48	MG161	CG161	VG801	VG802	VG	VG	VG	
culture +, JS133	MG162	**CG162**	VG806	**VG807**	VG808	VG809	VG810	
cagA+, s1a/m2	MG163	**CG163**	VG811	**VG812**	**VG813**	VG	VG	s1
	MG164	**CG164**	VG816	**VG817**	**VG818**	VG	VG	s1
	MG165	**CG165**	VG821	VG822	VG823	VG	VG	

		cagA	m1	m2	s1a	s1b	s2	us
ID 24	MG166	CG166	VG826	VG827	VG828	VG	VG	
culture +, JS134	MG167	**CG167**	VG831	VG832	**VG833**	VG	VG	**s1**
cagA+, s1a only	MG168	**CG168**	VG836	VG837	**VG838**	VG	VG	
	MG169	**CG169**	VG841	VG842	VG843	VG	VG	
	MG170	**CG170**	VG846	VG847	**VG848**	VG	VG	**s1**
6	MG171	CG171	**VG851**	VG852	VG853	VG854	VG855	
culture +, JS135	MG172	**CG172**	**VG856**	VG857	**VG858**	VG859	VG860	**s1**
cagA+, s1a/m1	MG173	**CG173**	**VG861**	VG862	**VG863**	VG864	VG865	**s1**
	MG174	**CG174**	**VG866**	VG867	**VG868**	VG869	VG870	**s1**
	MG175	**CG175**	**VG871**	VG872	VG873	VG874	VG875	**s1**
25	MG176	CG176	VG876	VG877	VG878	VG879	VG880	
culture +, JS136	MG177	**CG177**	**VG881**	**VG882**	**VG883**	VG884	VG885	**s1**
cagA-, s1a/m1	MG178	**CG178**	**VG886**	VG887	VG888	VG889	VG890	**s1**
	MG179	**CG179**	**VG891**	VG892	VG893	VG894	VG895	**s1**
	MG180	**CG180**	**VG896**	VG897	VG898	VG899	VG900	**s1**
ID 26	MG191	CG191	VG951	VG952	VG	VG	VG	
culture +, JS137	MG192	**CG192**	VG956	**VG957**	**VG958**	VG959	VG960	**s1**
cagA+, s1a/m2	MG193	**CG193**	VG961	**VG962**	**VG963**	VG	VG	**s1**
	MG194	**CG194**	VG966	**VG967**	**VG968**	VG	VG	**s1**
	MG195	**CG195**	VG971	**VG972**	**VG973**	VG	VG	**s1**
ID 49	MG196	CG196	VG976	VG977	VG978	VG	VG	
culture +, JS139	MG197	**CG197**	**VG981**	VG982	**VG983**	VG984	VG985	**s1**
cagA+,s1/m1	MG198	**CG198**	**VG986**	VG987	**VG988**	VG	VG	**s1**
	MG199	**CG199**	**VG991**	VG992	**VG993**	VG	VG	**s1**
	MG200	**CG200**	**VG996**	VG997	VG998	VG	VG	
ID 46	MG201	CG201	VG1001	VG1002	VG	VG	VG	
	MG202	CG202	VG1006	**VG1007**	VG	VG	VG	**s2**
	MG203	CG203	VG1011	**VG1012**	VG	VG	VG	**s2**
	MG204	CG204	VG1016	**VG1017**	VG	VG	VG	**s2**
	MG205	CG205	VG1021	**VG1022**	VG	VG	VG	**s2**
ID 47	MG221	CG221	VG1101	VG1102	VG1103			
	MG222	**CG222**	**VG1106**	VG1107	**VG1108**	VG1109	VG1110	**s1**
	MG223	**CG223**	**VG1111**	VG1112	**VG1113**			**s1**
	MG224	**CG224**	**VG1116**	VG1117	**VG1118**			**s1**
	MG225	**CG225**	**VG1121**	VG1122	**VG1123**			**s1**
ID 7	MG226	CG226	VG1126	VG1127	VG1128			
culture +, JS138	MG227	**CG227**	**VG1131**	VG1132	**VG1133**	VG1134	VG1135	**s1**
cagA+,s1a/m1	MG228	**CG228**	**VG1136**	VG1137	**VG1138**			**s1**
	MG229	**CG229**	**VG1141**	VG1142	**VG1143**			**s1**
	MG230	**CG230**	**VG1146**	VG1147	**VG1148**			**s1**

	code	cagA	m1	m2	s1a	s1b	s2	us
ID 20	MG231	**CG231**	VG1151	**VG1152**	VG1153			
	MG232	**CG232**	VG1156	**VG1157**	**VG1158**	VG1159	VG1160	**s1**
	MG233	**CG233**	VG1161	**VG1162**	**VG1163**			**s1**
	MG234	**CG234**	VG1166	**VG1167**	**VG1168**			**s1**
	MG235	**CG235**	VG1171	**VG1172**	VG1173			
ID 39	MG236	**CG236**	**VG1176**	VG1177	**VG1178**			**s1**
culture +, JS140	MG237	**CG237**	**VG1181**	VG1182	**VG1183**	VG1184	VG1185	**s1**
cagA+,s1a/m1	MG238	**CG238**	**VG1186**	VG1187	**VG1188**			**s1**
	MG239	**CG239**	**VG1191**	VG1192	**VG1193**			**s1**
	MG240	**CG240**	**VG1196**	VG1197	**VG1198**			**s1**
ID 40	MG246	CG246	VG1226	VG1227	VG1228			
culture +, JS141	MG247	**CG247**	VG1231	**VG1232**	**VG1233**	VG1234	VG1235	**s1**
cagA+, s1a/m2	MG248	**CG248**	VG1236	**VG1237**	**VG1238**			**s1**
	MG249	**CG249**	VG1241	**VG1242**	**VG1243**			**s1**
	MG250	**CG250**	VG1246	**VG1247**	**VG1247**			**s1**
ID 27	MG251	CG251	VG1251	VG1252	VG1253			
culture +, JS142	MG252	CG252	VG1256	**VG1257**	**VG1258**	VG1259	VG1260	**s1**
cagA-, s1a/m2	MG253	CG253	VG1261	**VG1262**	**VG1263**			**s1**
	MG254	CG254	VG1266	**VG1267**	**VG1268**			**s1**
	MG255	CG255	VG1271	**VG1272**	**VG1273**			**s1**
ID 31	MG256	**CG256**	VG1276	**VG1277**	**VG1278**			**s1**
culture +, JS148	MG257	**CG257**	VG1281	**VG1282**	**VG1283**	VG1284	VG1285	**s1**
cagA +, s1a/m2	MG258	**CG258**	VG1286	**VG1287**	**VG1288**			**s1**
	MG259	**CG259**	VG1291	**VG1292**	**VG1293**			**s1**
	MG260	**CG260**	VG1296	**VG1297**	VG1298			
ID 28	MG261	**CG261**	VG1301	VG1302	**VG1303**			**s1**
culture +, JS143	MG262	**CG262**	VG1306	VG1307	**VG1308**	VG1309	VG1310	**s1**
cagA+, s1a only	MG263	**CG263**	VG1311	VG1312	**VG1313**			**s1**
	MG264	**CG264**	VG1316	VG1317	**VG1318**			**s1**
	MG265	**CG265**	VG1321	VG1322	**VG1323**			**s1**
ID 30	MG266	CG266	VG1326	VG1327	VG1328			
culture +, JS147	MG267	**CG267**	VG1331	**VG1332**	**VG1333**	**VG1334**	VG1335	**s1**
cagA +, s1a/m2	MG268	**CG268**	VG1336	**VG1337**	**VG1338**			**s1**
	MG269	**CG269**	VG1341	**VG1342**	**VG1343**			**s1**
	MG270	**CG270**	VG1346	**VG1347**	**VG1348**			**s1**
ID 29	MG271	CG271	VG1351	**VG1352**	**VG1353**			**s1**
culture +, JS145	MG272	CG272	VG1356	**VG1357**	**VG1358**	VG1359	VG1360	**s1**
cagA -, s1a/m2	MG273	CG273	VG1361	**VG1362**	**VG1363**			**s1**
	MG274	CG274	VG1366	**VG1367**	**VG1368**			**s1**
	MG275	CG275	VG1371	**VG1372**	**VG1373**			**s1**

	code	cagA	m1	m2	s1a	s1b	s2	us
ID 8	MG281	CG281	VG1401	VG1402	VG1403			s1
culture +, JS144	MG282	CG282	VG1406	VG1407	VG1408	VG1409	VG1410	s1
cagA +, s1a/m2	MG283	CG283	VG1411	VG1412	VG1413			s1
	MG284	CG284	VG1416	VG1417	VG1418			s1
	MG							
ID 21	MG286	CG286	VG1426	VG1427	VG1428			
	MG287	CG287	VG1431	VG1432	VG1433	VG1434	VG1435	s1
	MG288	CG288	VG1436	VG1437	VG1438			s1
	MG289	CG289	VG1441	VG1442	VG1443			s1
	MG290	CG290	VG1446	VG1447	VG1448			s1
ID 10	MG291	CG291	VG1451	VG1452	VG1453			s1
culture +, JS149	MG292	CG292	VG1456	VG1457	VG1458	VG1459	VG1460	s1
cagA +, s1a/m2	MG293	CG293	VG1461	VG1462	VG1463			s1
	MG294	CG294	VG1466	VG1467	VG1468			s1
	MG295	CG295	VG1471	VG1472	VG1473			s1
ID 9	MG296	CG296	VG1476	VG1477	VG1478			
culture +, JS146	MG297	CG297	VG1481	VG1482	VG1483	VG1484	VG1485	s1
cagA +, s1a/m2	MG298	CG298	VG1486	VG1487	VG1488			s1
	MG299	CG299	VG1491	VG1492	VG1493			
	MG300	CG300	VG1496	VG1497	VG1498			s1
ID 33	MG306	CG306	VG1526	VG1527	VG1528			
culture +, JS151	MG307	CG307	VG1531	VG1532	VG1533	VG1534	VG1535	s1
cagA +, s1/m2	MG308	CG308	VG1536	VG1537	VG1538			s1
	MG309	CG309	VG1541	VG1542	VG1543			s1
	MG310	CG310	VG1546	VG1547	VG1548			s1
ID 37	MG311	CG311	VG1551	VG1552	VG1553			
	MG312	CG312	VG1556	VG1557	VG1558	VG1559	VG1560	s1
	MG313	CG313	VG1561	VG1562	VG1563			s1
	MG314	CG314	VG1566	VG1567	VG1568			s1
	MG315	CG315	VG1571	VG1572	VG1573			s1
ID 32	MG316	CG316	VG1576	VG1577	VG1578			
culture +, JS150	MG317	CG317	VG1581	VG1582	VG1583	VG1584	VG1585	s1
cagA +, s1/m2	MG318	CG318	VG1586	VG1587	VG1588			s1
	MG319	CG319	VG1591	VG1592	VG1593			s1
	MG320	CG320	VG1596	VG1597	VG1598			s1
ID 22	MG321	CG321	VG1601	VG1602	VG1603			
	MG322	CG322	VG1606	VG1607	VG1608	VG1609	VG1610	s1
	MG323	CG323	VG1611	VG1612	VG1613			s1
	MG324	CG324	VG1616	VG1617	VG1618			s1
	MG325	CG325	VG1621	VG1622	VG1623			s1

	CODE	cagA	m1	m2	s1a	s1b	s2	us
ID 41	MG331	CG331	VG1651	VG1652	VG1653			
culture +, JS152	MG332	**CG332**	VG1656	**VG1657**	**VG1658**	VG1659	VG1660	**s1**
cagA +, s1a/	MG333	**CG333**	VG1661	**VG1662**	**VG1663**			**s1**
	MG334	**CG334**	VG1666	**VG1667**	**VG1668**			**s1**
	MG335	**CG335**	VG1671	**VG1672**	**VG1673**			**s1**
ID 38	MG341	CG341	VG1701	VG1702	VG1703			
	MG342	**CG342**	VG1706	**VG1707**	**VG1708**	VG1709	VG1710	
	MG343	**CG343**	VG1711	**VG1712**	**VG1713**			**s1**
	MG344	CG344	VG1716	**VG1717**	VG1718			
	MG345	**CG345**	VG1721	**VG1722**	VG1723			
ID 34	MG346	CG346	VG1726	VG1727	VG1728			
culture +, JS153	MG347	CG347	VG1731	**VG1732**	**VG1733**	VG1734	VG1735	**s1**
cagA -, s1a/m2	MG348	CG348	VG1736	**VG1737**	**VG1738**			**s1**
	MG349	**CG349**	VG1741	**VG1742**	**VG1743**			**s1**
	MG350	**CG350**	VG1746	**VG1747**	**VG1748**			**s1**
ID 11	MG351	**CG351**	VG1751	**VG1752**	**VG1753**			**s1**
culture +, JS154	MG352	**CG352**	VG1756	**VG1757**	**VG1758**	VG1759	VG1760	**s1**
cagA +, s1a/m2	MG353	**CG353**	VG1761	**VG1762**	**VG1763**			**s1**
	MG354	**CG354**	VG1766	**VG1767**	**VG1768**			**s1**
	MG355	**CG355**	VG1771	**VG1772**	**VG1773**			**s1**

A 5.2 Supplementary data for Chapter 4

A5.2.1 ALL PATIENTS

	Grade of Bacterial Density			Grade of Acute Inflammation			Grade of Chronic Inflammation		
	0/1	**2/3**	**TOTAL**	**0/1**	**2/3**	**TOTAL**	**0/1**	**2/3**	**TOTAL**
cagA +/ s1/m1	16	23	**39**	18	21	**39**	10	29	**39**
cagA –/ s1/m1	4	2	**6**	4	2	**6**	2	4	**6**
cagA +/ s1/m2	13	27	**40**	17	23	**40**	3	37	**40**
cagA –/ s1/m2	10	2	**12**	9	3	**12**	4	8	**12**
cagA +/ s2/m2	2	1	**3**	3	0	**3**	2	1	**3**
cagA –/ s2/m2	6	4	**10**	7	3	**10**	6	4	**10**
cagA +/ s1	3	5	**8**	4	4	**8**	0	8	**8**
cagA –/ s1	1	0	**1**	1	0	**1**	1	0	**1**
cagA +/m2	0	1	**1**	0	1	**1**	0	1	**1**
TOTAL	**55**	**65**	**120**	**63**	**57**	**120**	**28**	**92**	**120**

A5.2.2 NUD PATIENTS

	Grade of Bacterial Density			Grade of Acute Inflammation			Grade of Chronic Inflammation		
	0/1	**2/3**	**TOTAL**	**0/1**	**2/3**	**TOTAL**	**0/1**	**2/3**	**TOTAL**
cagA +/ s1/m1	8	13	**21**	13	8	**21**	6	15	**21**
cagA –/ s1/m1	2	2	**4**	2	2	**4**	2	2	**4**
cagA +/ s1/m2	7	16	**23**	12	11	**23**	2	21	**23**
cagA –/ s1/m2	7	2	**9**	7	2	**9**	4	5	**9**
cagA +/ s2/m2	2	1	**3**	3	0	**3**	2	1	**3**
cagA –/ s2/m2	5	4	**9**	6	3	**9**	5	4	**9**
cagA +/ s1	2	2	**4**	1	3	**4**	0	4	**4**
cagA –/ s1	0	0	**0**	0	0	**0**	0	0	**0**
cagA +/m2	0	1	**1**	0	1	**1**	0	1	**1**
TOTAL	**33**	**41**	**74**	**44**	**30**	**74**	**21**	**53**	**74**

A5.2.3 PUD PATIENTS

	Grade of Bacterial Density			Grade of Acute Inflammation			Grade of Chronic Inflammation		
	0/1	**2/3**	**TOTAL**	**0/1**	**2/3**	**TOTAL**	**0/1**	**2/3**	**TOTAL**
cagA +/ s1/m1	6	10	**16**	3	13	**16**	3	13	**16**
cagA –/ s1/m1	2	0	**2**	2	0	**2**	0	2	**2**
cagA +/ s1/m2	6	9	**15**	5	10	**15**	1	14	**15**
cagA –/ s1/m2	3	0	**3**	2	1	**3**	0	3	**3**
cagA +/ s2/m2	0	0	**0**	0	0	**0**	0	0	**0**
cagA –/ s2/m2	1	0	**1**	1	0	**1**	1	0	**1**
cagA +/ s1	1	2	**3**	2	1	**3**	0	3	**3**
cagA –/ s1	1	0	**1**	1	0	**1**	1	0	**1**
cagA +/m2	0	0	**0**	0	0	**0**	0	0	**0**
TOTAL	**20**	**21**	**41**	**16**	**25**	**41**	**6**	**35**	**41**

A5.3 Supplementary details for Chapter 5

A5.3.1 Complete Helicoblot 2.0 data

code	Bacteria	Pathol	*cagA*	*vacA*	116KDa	89KDa	35KDa	30KDa	26KDa	19KDa
36		DU	P	s1a/m2	WP	N	N	N	P	P
37		DU	P	s1a	P	P	N	P	P	P
31	JS148	DU	P	s1a/m2	P	P	P	P	P	N
25	JS136	DU	P	s1a/m1 +m2	P	P	N	P	P	P
24	JS134	DU	P	s1a	P	N	P	P	P	P
32	JS150	DU	P	s1a/m2	P	N	P	N	P	P
38		DU	P	s1a/m2	P	P	N	P	P	P
33	JS151	DU	P	s1a/m2	P	N	P	P	P	N
28	JS143	DU	P	s1a/m1	P	P	P	P	P	N
26	JS137	DU	P	s1a/m2	P	P	N	P	P	P
35		DU	N	s1a/m1	N	N	N	P	P	N
29	JS145	DU	N	s1a/m2	N	N	N	N	N	N
30	JS147	DU	P	s1a+b/ m2	P	N	N	P	P	N
23	JS130	DU	P	s1a/m2	P	P	P	N	P	N
43		GU	P	s1a/m1	N	N	N	N	P	N
39	JS140	GU	P	s1a/m1	P	P	P	P	P	P
40	JS141	GU	P	s1a/m2	P	P	P	P	P	N
46		GU	N	s2/m2	N	N	N	N	N	N
47		GU	P	s1a/m1	P	P	N	P	P	N
44		GU	P	s1b/m1	P	P	P	P	P	N
45		GU	P	s1b/m1	P	P	P	N	P	P
50		CA	P	s1b/m1	P	N	N	P	P	N
49	JS139	CA	P	s1a/m1	P	N	P	N	P	P
51		CA	P	s1a	P	P	P	P	P	N
52		CA	P	s1b/m1	P	N	P	N	N	N
48	JS133	CA	P	s1a/m2	P	P	N	P	N	P
10	JS149	N	P	s1a/m2	P	N	N	N	P	N
14		N	N	s2/m2	P	N	N	P	N	N
13		N	P	s1a/m2	N	N	N	N	N	N
22		N	P	s1a/m2	P	P	P	P	P	P
6	JS135	N	P	s1a/m1	P	N	N	P	P	P
8	JS144	N	P	s1a/m2	P	N	N	P	P	N
15		N	P	s1a/m1	P	N	P	N	P	N
4	JS131	N	N	s1a/m2	N	N	N	N	P	P
17		N	P	s1a/m2	P	N	N	P	P	P
9	JS146	N	P	s1a/m2	N	N	P	P	N	N
19		N	P	s1b/m2	P	P	N	N	P	N
20		N	P	s1a/m2	P	P	N	N	P	N
5	JS132	N	N	s2/m2	P	N	N	P	P	N
21		N	P	s1a/m1	P	N	N	P	P	P
11	JS154	N	P	s1a/m2	P	N	N	P	P	N

A5.4 Supplementary details for Chapter 6

A5.4.1 Details of p120 ELISA with titre values (580 patients)

Sample	ELISA CODE	ELISA	Sex	Age	p120 UNITS
1	1	0.136	F	76	2.2
3	2	0.488	F	71	3.8
9	2	0.557	F	71	7.2
13	1	0.231	M	65	2.2
14	1	0.169	M	69	9.4
16	1	0.100	F	70	3.6
20	2	0.427	M	67	5.7
22	2	0.306	M	71	33.6
24	1	0.100	M	70	1.7
26	2	0.406	F	69	3.0
27	2	0.467	M	52	35.2
28	2	0.480			4.2
31	1	0.197	M	43	4.5
32	2	0.255	M	73	0.9
33	2	0.396	M	71	0.4
34	2	0.464			12.3
35	1	0.117	M	76	0.0
36	1	0.071	M	76	6.0
38	2	0.491	M	57	4.7
41	2	0.301			3.0
42	2	0.392			1.9
43	2	0.504			2.8
46	2	0.360	M	63	1.1
48	1	0.077	M	75	1.5
51	2	0.507	F	81	28.5
52	1	0.194	M	77	2.9
55	2	0.492	M	68	2.5
59	2	0.357	F	70	22.8
60	1	0.103	M	61	0.1
61	2	0.252	M	85	3.8
62	2	0.322	M	39	13.3
64	2	0.573	F	72	33.8
66	1	0.188	M	51	3.3
69	2	0.423	M	70	5.0
70	1	0.114	M	62	2.5
74	2	0.266	M	70	5.0
75	1	0.113	F	63	1.0
76	1	0.106	M	66	1.2
77	1	0.130	M	62	1.1
78	2	0.564	M	84	2.5
81	1	0.152	F	67	0.8
82	2	0.349	M	73	3.3
83	1	0.120	F	65	1.3
84	2	0.245	M	74	13.4
85	2	0.577	M	62	1.9
91	2	0.401	M	59	15.9
96	2	0.256	M	61	6.2

97	2	0.460	M	46	23.4
98	2	0.448	M	71	6.8
99	2	0.421	M	79	16.7
100	1	0.085	F	69	0.2
101	2	0.604	M	72	3.5
111	1	0.081	F	34	0.4
112	2	0.471	F	78	5.5
113	1	0.226	M	76	14.2
115	1	0.115	M	53	3.3
117	2	0.520	M	76	15.1
119	2	0.359	F	44	14
120	2	0.400			1.9
121	1	0.234	M	79	3.7
127	2	0.460			2.7
131	2	0.329	F	40	21.4
132	1	0.135	M	62	4.9
133	2	0.561	M	65	16.9
135	1	0.141	F	68	1
136	2	0.440	M	66	11.7
138	2	0.268	M	44	14.8
142	1	0.087	F	80	0
145	1	0.112	F	83	0.5
146	2	0.403			7.9
148	1	0.187	M	67	2.7
150	1	0.144	M	50	2.2
151	1	0.067	M	64	0.3
155	1	0.080	M	69	0.6
164	1	0.154	M	54	1.8
165	1	0.200	M	85	10.5
166	2	0.279	F	72	8
167	1	0.171	F	69	1.2
171	1	0.178	M	67	0
172	2	0.300			2.5
173	1	0.068	M	74	2.5
176	1	0.100	M	56	1.0
179	1	0.150	M	42	1.8
180	2	0.407	M	54	9.1
181	2	0.381	M	42	1.1
183	2	0.513	M	62	3.6
184	2	0.423	F	88	8.1
189	1	0.092	F	76	3.3
190	1	0.076	F	72	1.5
195	2	0.369	M	69	1.8
196	2	0.428	M	71	4.2
197	1	0.203	M	63	17.4
199	1	0.096	M	66	1.5
203	1	0.181	M	45	1.1
205	2	0.268	F	55	7.1
206	2	0.140	M	84	2.9
208	2	0.492	M	58	17.9
209	1	0.206	M	71	10.0
213	2	0.410			1.3

214	2	0.520	M	62	1.7
215	2	0.286			20.5
220	1	0.190	M	70	1.2
223	1	0.173	M	56	1.2
227	1	0.234	F	73	1.0
231	1	0.136	M	57	1.5
236	1	0.187	M	83	0.2
237	1	0.221	M	48	7.7
238	2	0.468	F	63	20.5
242	2	0.360	F	84	8
243	2	0.264			1.6
244	2	0.588	F	70	33
245	2	0.426	F	80	14.7
250	2	0.527	M	38	37.2
251	2	0.395			1.7
252	2	0.374	M	80	8.1
256	1	0.140	M	75	4.0
260	1	0.195	M	51	5.5
261	2	0.352	M	83	4.1
262	2	0.427			1.6
268	2	0.499	M	67	32.6
269	2	0.503	M	84	6.3
271	2	0.469	M	63	2.7
274	2	0.440	M	55	16.3
275	1	0.114	M	69	0.9
276	2	0.496	F	68	28.4
278	1	0.083	F	68	2.8
279	2	0.306			2.5
281	2	0.449	F	70	19.7
284	2	0.497	M	53	30.5
285	2	0.472	M	76	20.1
286	2	0.258	M	81	6.7
289	1	0.197	M	81	0.8
291	2	0.275	M	69	3.3
293	2	0.265	M	58	2.9
294	1	0.061	F	87	2
295	2	0.247	M	58	3.8
296	2	0.312	F	62	5.7
297	2	0.345	M	58	10.8
299	2	0.393	M	63	22.2
301	1	0.230	M	67	1.5
303	2	0.323	F	68	1.2
304	2	0.459	M	70	11.7
305	2	0.397	M	44	11.4
306	1	0.092	M	77	3.8
307	2	0.342	M	45	2.7
310	2	0.344	F	80	7.9
313	1	0.189	M	61	2
316	2	0.344	M	57	11.8
317	2	0.460	F	66	12.8
318	2	0.368	F	84	3.7
320	2	0.523	M	73	1.6

322	2	0.400	M	60	7.8
323	2	0.295	M	57	1.8
324	1	0.239	F	75	18.3
327	2	0.362	F	39	1.9
331	2	0.407	M	41	13.3
332	1	0.175	F	63	2.2
333	1	0.117	M	37	3.3
334	2	0.290	M	75	1.5
335	2	0.292	F	79	14.0
338	2	0.533	F	64	14.3
340	2	0.251	M	53	4.1
343	2	0.289	M	61	1.1
345	2	0.256	M	69	2.5
346	2	0.523	M	64	31.1
347	1	0.081	M	57	2.9
350	2	0.327	M	55	1.0
353	2	0.548	F	88	2.6
354	2	0.450	M	63	2.5
356	2	0.531	F	80	11.2
362	1	0.110	M	67	5.2
363	2	0.336	F	71	8.7
364	2	0.424	F	67	3.5
366	1	0.101	M	63	1.4
369	1	0.092	M	70	0.9
370	1	0.107	F	62	1.8
372	2	0.378	M	64	2.1
373	1	0.144	M	60	2.9
375	1	0.198	M	61	2.3
376	1	0.167	M	70	1.5
377	2	0.271	M	60	9.4
379	1	0.113	F	71	4.1
382	2	0.427	F	85	23.1
383	1	0.109	F	88	5.3
385	2	0.326	M	72	2.1
386	1	0.076	F	69	0.8
389	2	0.318	M	60	2.9
390	1	0.085	M	68	1.3
391	1	0.219	M	52	5.6
392	1	0.098	M	64	2.3
394	1	0.140	M	56	1.5
395	2	0.366	M	65	1.8
397	2	0.329	M	45	16.7
398	2	0.336	F	52	15.2
400	2	0.270	M	64	2.2
401	2	0.402	M	68	16.8
402	2	0.372	M	69	8.2
403	1	0.199	M	79	3.1
404	2	0.393	F	79	19.8
407	2	0.382	M	59	18.5
410	2	0.407	M	80	18.3
411	1	0.218	M	77	6.7
412	2	0.345	F	72	24.6

416	1	0.161	M	61	4.2
417	1	0.117	M	82	3.9
419	2	0.329	M	70	6.6
422	2	0.272	M	51	2.9
424	1	0.179	F	65	4.0
425	1	0.118	M	43	7.0
426	1	0.211	M	75	3.9
429	1	0.181	F	64	3.6
431	1	0.148	M	48	3.4
432	2	0.389	M	58	14.4
433	2	0.386	M	68	23.1
437	1	0.208	M	72	6.3
439	1	0.120	F	84	3.9
440	1	0.150	F	60	3.1
444	1	0.114	M	54	4.7
446	1	0.165	M	54	3.3
450	2	0.324	F	77	32.0
457	1	0.201	M	72	4.5
460	2	0.379	M	65	13.2
468	2	0.408	M	63	1.5
473	1	0.234	M	57	5.4
478	1	0.085	F	74	4.2
480	1	0.079	M	47	4.1
483	1	0.178	F	78	3.6
484	2	0.367	F	84	4.1
485	1	0.221	M	69	5.6
486	2	0.249	F	68	3.1
487	1	0.162	M	64	4.5
489	2	0.478	M	67	30.5
490	2	0.465	M	53	3.3
491	1	0.110	F	66	1.7
494	1	0.212	M	58	5.3
499	1	0.131	M	55	4.2
504	2	0.430	M	53	5.8
505	2	0.338	F	81	4.2
507	2	0.439	F	68	19.7
509	1	0.184	F	79	3.3
511	2	0.260	F	61	2.9
512	1	0.136	M	59	3.2
515	2	0.442	M	85	17.3
516	1	0.078	M	60	4.8
517	2	0.377	F	61	4.6
523	2	0.457	F	80	5.1
525	2	0.430	F	82	2.9
530	2	0.472	F	70	19.7
535	1	0.173	M	54	6.8
539	2	0.243	M	69	10.7
542	2	0.530	F	86	2.5
543	1	0.190	F	62	1.6
545	2	0.418	M	70	2.6
546	2	0.458	M	49	19.0
553	2	0.436	M	51	30.4

556	2	0.318	M	57	17.7
558	1	0.126	F	93	0.3
563	2	0.294	M	72	10.6
564	2	0.494	M	65	31.0
566	2	0.477	F	59	16.7
569	2	0.249	M	77	4.0
570	2	0.287	M	67	23.2
571	1	0.127	F	72	2.7
573	2	0.398	F	74	1.7
575	2	0.269	M	64	1.5
576	2	0.270	M	64	0.7
577	2	0.464	F	58	15.0
578	1	0.231	M	74	2.1
579	1	0.182	F	45	1.1
580	1	0.096	F	68	2.8
581	2	0.410	M	56	4.1
582	1	0.097	M	61	2.7
583	2	0.244	F	59	1.9
584	1	0.159	M	57	2.8
585	2	0.245	M	59	2.6
586	2	0.341	F	71	5.9
588	2	0.286	M	57	4.2
1001	2	0.357	F	60	12.1
1003	1	0.237	M	86	2.7
1008	1	0.224	F	84	3.7
1015	2	0.469	M	61	34.2
1016	2	0.524	F	71	1.9
1017	1	0.158	M	65	3.9
1028	1	0.090	M	85	2.7
1029	1	0.148	M	60	2.4
1039	2	0.487	M	46	3.0
1040	2	0.409	M	60	2.9
1043	2	0.267	F	69	2.3
1044	2	0.382	M	81	1.8
1049	1	0.072	M	38	2.2
1050	2	0.274	F	69	6.5
1054	1	0.133	M	73	3.2
1058	2	0.328	M	31	2.5
1059	2	0.434	M	71	1.6
1068	2	0.328	M	60	11.6
1076	1	0.227	M	72	3.7
1078	2	0.507	M	71	7.0
1084	2	0.308	M	60	2.8
1086	2	0.278	M	78	3.0
1102	2	0.294	F	58	2.5
1105	2	0.370	M	60	3.2
1106	2	0.274	F	62	1.0
1114	2	0.249	F	73	5.8
1127	2	0.397	M	72	13.3
1134	2	0.533	M	77	9.9
1145	2	0.480	M	77	7.8
1146	2	0.281			1.9

1154	1	0.227	F	62	0.9
1165	2	0.488	M	57	14.7
1181	1	0.142	F	50	4.5
1182	2	0.443	M	61	22.2
1183	1	0.130	M	37	0.7
1196	2	0.442	M	57	13.5
1201	2	0.420	M	55	17.3
1207	1	0.127	M	49	1.9
1209	2	0.479	M	52	24.3
1210	1	0.120	M	64	1.7
1221	2	0.256	M	68	6.6
1222	2	0.270	M	65	1.9
1234	1	0.238	F	86	3.5
1239	2	0.292	F	82	15.0
1240	2	0.442	F	55	3.0
1246	1	0.109	M	71	2.4
1262	2	0.240	M	56	8.2
1268	1	0.114	F	68	0.6
1271	1	0.179	M	60	5.3
1274	1	0.182	M	84	2.1
1279	2	0.428	F	82	8.4
1280	1	0.083	F	70	1.1
1286	2	0.478	M	92	10.5
1304	2	0.289	F	80	4.5
1305	1	0.106	F	74	2.1
1322	2	0.531	M	75	0.8
1326	1	0.205	M	51	3.8
1331	2	0.481	M	86	3.7
1333	2	0.304	M	49	11.2
1363	2	0.391	F	77	3.3
1369	1	0.179	F	74	4.4
1373	1	0.136	M	84	6.0
1378	2	0.327	M	71	5.1
1392	2	0.367	F	75	11.5
1400	2	0.523	M	73	2.0
2001	1	0.130			3.9
2002	1	0.144			3.1
2003	2	0.369			28.2
2004	2	0.346			10.8
2005	2	0.366			4.5
2006	2	0.246			5.3
2007	2	0.287			3.6
2008	2	0.381			19.0
2009	2	0.264			5.1
2010	2	0.487			32.5
2011	1	0.110			3.2
2012	2	0.384			3.0
2013	2	0.270			2.2
2014	2	0.348			1.2
2015	2	0.324			1.2
2016	2	0.323			2.5
2017	1	0.163			2.0

2018	1	0.185			0.5
2019	1	0.135			1.0
2020	2	0.467			2.1
2021	1	0.214			3.6
2022	1	0.154			4.7
2023	2	0.395			4.3
2024	2	0.296			1.7
2025	1	0.192			2.1
2026	2	0.287			3.8
2027	1	0.183			2.5
2028	1	0.145			1.4
2029	2	0.332			4.0
2030	1	0.125			12.4
2031	1	0.147			4.4
2032	1	0.099			1.6
2033	2	0.473			4.4
2034	1	0.137			2.9
2035	1	0.206			1.5
2036	2	0.529			8.3
2037	1	0.238			4.4
2038	2	0.433			28.8
2039	2	0.280			2.4
2040	1	0.175			3.9
2041	2	0.519			11.8
2042	2	0.522			14.6
2043	1	0.183			4.6
2044	1	0.127			3.0
2045	1	0.130			3.9
2046	2	0.376			6.5
2047	2	0.406			15.9
2048	1	0.128			2.3
2049	1	0.143			3.0
2050	1	0.178			3.7
2051	1	0.200			3.7
2052	2	0.319			7.1
2053	2	0.353			10.8
2054	1	0.228			5.7
2055	1	0.227			6.0
2056	1	0.212			2.7
2057	2	0.331			1.7
2058	2	0.241			2.6
2059	1	0.158			2.3
2060	2	0.274			13.2
2061	2	0.280			4.5
2062	2	0.510			32.2
2063	2	0.281			4.4
2064	2	0.415			4.1
2065	2	0.263			3.2
2066	1	0.227			1.2
2067	1	0.129			2.0
2068	2	0.257			2.9
2069	1	0.198			2.2

2070	1	0.142			2.6
2071	2	0.264			4.7
2072	2	0.536			36.7
2073	2	0.386			9.4
2074	1	0.228			3.1
2075	2	0.403			4.7
2076	2	0.336			10.9
2077	1	0.173			3.9
2078	2	0.375			1.3
2079	2	0.354			5.9
2080	2	0.322			4.4
2081	2	0.430			7.7
2082	2	0.247			1.3
2083	2	0.311			2.8
2084	1	0.173			2.8
2085	1	0.226			3.1
2086	2	0.464			1.2
2087	2	0.336			12.1
2088	1	0.209			3.3
2089	2	0.306			3.6
2090	1	0.213			3.2
2091	1	0.145			3.0
2092	2	0.466			5.8
2093	2	0.477			9.1
2094	2	0.330			7.6
2095	2	0.344			6.3
2096	2	0.434			37.1
2097	2	0.261			4.0
2098	2	0.427			21.9
2099	2	0.250			16.5
2100	1	0.212			4.6
2101	2	0.501			5.1
2102	2	0.484			5.3
2103	1	0.160			1.3
2104	1	0.176			2.0
2105	1	0.123			1.0
2106	1	0.138			3.5
2107	1	0.122			1.5
2108	1	0.200			2.6
2109	1	0.156			2.4
2110	1	0.194			2.8
2111	1	0.176			2.9
2112	2	0.427			2.9
2113	2	0.482			2.8
2114	1	0.136			1.6
2115	1	0.224			2.3
2116	1	0.150			0.9
2117	1	0.155			1.2
2118	2	0.303			2.4
2119	1	0.142			1.7
2120	2	0.459			29.8
2121	1	0.148			13.7

2122	2	0.395			20.5
2123	1	0.180			2.8
2124	1	0.186			3.5
2125	2	0.260			2.3
2126	2	0.383			3.1
2127	2	0.396			6.8
2128	1	0.128			3.4
2129	2	0.293			3.5
2130	1	0.111			1.6
2131	1	0.229			4.9
2132	2	0.429			19.5
2133	1	0.197			2.7
2134	2	0.473			2.8
2135	2	0.404			8.4
2136	2	0.468			5.4
2137	2	0.455			5.4
2138	2	0.450			5.9
2139	1	0.169			3.8
2140	1	0.141			2.2
2141	2	0.255			12.8
2142	2	0.349			3.7
2143	1	0.223			5.3
2144	2	0.356			23.9
2145	1	0.168			3.5
2146	2	0.312			3.0
2147	1	0.110			2.6
2148	1	0.207			5.0
2149	2	0.275			3.7
2150	1	0.218			2.2
2151	2	0.361			9.4
2152	2	0.258			4.1
2153	2	0.249			2.3
2154	1	0.198			11.4
2155	1	0.130			3.0
2156	2	0.297			3.7
2157	1	0.184			3.9
2158	2	0.384			8.0
2159	2	0.311			7.5
2160	2	0.348			5.5
2161	2	0.303			5.2
2162	1	0.220			1.9
2163	2	0.324			3.5
2164	2	0.425			34.8
2165	2	0.421			31.0
2166	1	0.158			3.4
2167	2	0.265			5.6
2168	2	0.353			9.9
2169	1	0.211			4.5
2170	1	0.239			4.9
2171	1	0.168			3.7
2172	2	0.290			4.0
2173	2	0.327			4.4

2174	1	0.169			4.8
2175	1	0.138			2.7
2176	2	0.462			32.2
2177	1	0.192			1.2
2178	2	0.469			3.3
2179	1	0.150			6.0
2180	2	0.502			28.7
2181	1	0.178			2.9
2182	1	0.114			3.4
2183	2	0.428			25.1
2184	1	0.188			3.6
2185	2	0.395			5.8
2186	1	0.154			6.3
2187	2	0.448			17.9
2188	2	0.471			5.1
2189	2	0.325			24.2
2190	1	0.193			1.9
2191	1	0.174			3.0
2192	1	0.135			1.0
2193	1	0.232			1.3
2194	2	0.255			8.6
2195	1	0.189			1.4
2196	2	0.398			10.5
2250	1	0.166			0.9
2251	2	0.465			2.3
2252	2	0.507			1.2
2253	2	0.325			17.1
2254	2	0.325			1.5
2255	1	0.195			1.6
2256	2	0.357			1.8
2257	1	0.161			0.5
2258	2	0.263			1.2
2259	2	0.271			1.7
2260	2	0.410			8.4
2261	1	0.153			1.3
2262	1	0.201			1.4
2263	2	0.284			1.5
2264	2	0.434			17.4
2265	2	0.286			18.2
2266	2	0.420			2.0
2267	1	0.163			1.4
2268	1	0.206			1.5
2269	1	0.198			1.1
2270	1	0.236			1.8
2271	1	0.225			1.7
2272	2	0.438			6.0
2273	1	0.166			1.7
2274	2	0.261			1.6
2275	2	0.257			2.8
2276	2	0.486			2.7
2277	1	0.219			9.4
2278	2	0.389			22.8

2279	2	0.274			1.1
2280	1	0.214			3.3
2281	2	0.413			13.1
2282	2	0.476			1.5
2283	2	0.387			24.3
2284	2	0.363			1.4
2285	2	0.486			13.6
2286	1	0.160			1.6
2287	2	0.326			16.7
2288	1	0.215			2.5
2289	2	0.329			2.6
2290	2	0.409			1.3
2291	2	0.515			1.5

A5.4.2 Immunoblot details of 207 MI bloods

p120 UNITS	Sample	ELISA	Sex	Age	Blot	Anti-CagA Ab	p120 ELISA
37.2	250	0.527	M	48	pos	pos	1.750
35.2	27	0.467	M	51	pos	pos	1.656
33.8	64	0.573	F	71	pos	pos	1.592
33.6	22	0.306	M	69	pos	pos	1.580
33.0	244	0.588	F	70	pos	pos	1.554
32.6	268	0.499	M	67	pos	pos	1.532
31.1	346	0.523	M	63	pos	pos	1.670
30.5	284	0.497	M	52	pos	pos	1.638
28.5	51	0.507	F	79	pos	pos	1.340
28.4	276	0.496	F	68	pos	pos	1.334
24.6	412	0.345	F	71	pos	pos	1.220
23.4	97	0.460	M	45	pos	pos	1.260
23.1	382	0.427	F	85	pos	pos	1.142
22.8	59	0.357	F	68	pos	pos	1.009
22.2	299	0.393	M	62	pos	pos	1.195
21.4	131	0.329	F	40	pos	pos	1.005
20.5	238	0.468	F	67	pos	pos	0.965
20.1	285	0.472	M	75	pos	pos	1.079
19.8	404	0.393	F	78	pos	pos	0.982
19.7	281	0.449	F	68	pos	pos	1.058
18.5	407	0.382	M	58	pos	pos	0.917
18.3	410	0.407	M	79	pos	pos	0.906
17.9	208	0.492	M	58	pos	pos	0.842
17.4	197	0.203	M	62	pos	pos	0.936
16.9	133	0.561	M	65	pos	pos	0.796
16.8	401	0.402	M	61	pos	pos	0.832
16.7	99	0.421	M	78	pos	pos	0.897
16.7	397	0.329	M	44	pos	pos	0.829
16.3	274	0.440	M	55	pos	pos	0.769
15.9	91	0.401	M	57	pos	pos	0.854
15.2	398	0.336	F	51	pos	pos	0.754
15.1	117	0.520	M	75	pos	pos	0.809
14.8	138	0.268	M	43	pos	pos	0.694
14.7	245	0.426	F	79	pos	pos	0.694
14.3	338	0.533	F	63	pos	pos	0.768
14.2	113	0.226	M	73	pos	pos	0.764
14.0	119	0.359	F	44	pos	pos	0.660
14.0	335	0.292	F	78	pos	pos	0.750
13.3	62	0.322	M	38	pos	pos	0.716
13.3	331	0.407	M	39	pos	pos	0.713
12.8	317	0.460	F	65	pos	pos	0.686
11.8	316	0.344	M	56	pos	pos	0.634
11.7	136	0.44	M	66	pos	pos	0.550
11.7	304	0.459	M	69	pos	pos	0.629
11.4	305	0.397	M	43	pos	pos	0.612
11.2	356	0.531	F	79	pos	pos	0.601
10.8	297	0.345	M	67	pos	pos	0.583
10.5	165	0.200	M	85	pos	pos	0.494
9.4	14	0.169	M	68	weak	pos	0.444
9.4	377	0.271	M	60	pos	pos	0.467
9.1	180	0.407	M	54	pos	pos	0.427
8.7	363	0.336	F	60	neg	neg	0.468
8.2	402	0.372	M	69	pos	pos	0.408
8.1	184	0.423	F	88	pos	pos	0.380
8.1	252	0.374	M	80	pos	pos	0.382
8.0	166	0.279	F	72	pos	pos	0.375
8.0	242	0.360	F	84	pos	pos	0.375
7.8	322	0.400	M	59	pos	pos	0.420
7.7	237	0.221	M	63	pos	pos	0.412

p120 UNITS	Sample	ELISA	Sex	Age	Blot	Anti-CagA Ab	p120 ELISA
7.5	2159	0.311	M	78	pos	pos	0.368
7.2	9	0.557	F	70	pos	pos	0.340
7.1	205	0.268	F	55	pos	pos	0.334
7.1	425	0.118	M	42	neg	pos	0.348
7.1	2052	0.319	F	45	pos	pos	0.340
7.0	1078	0.507	M	69	pos	pos	0.351
6.8	98	0.448	M	70	pos	pos	0.367
6.8	535	0.173	M	53	weak	pos	0.336
6.8	2127	0.396	M	47	pos	pos	0.333
6.7	286	0.258	M	80	pos	pos	0.358
6.7	411	0.218	M	75	pos	pos	0.333
6.6	419	0.329	M	69	pos	pos	0.327
6.6	1221	0.256	M	67	pos	pos	0.333
6.5	1050	0.274	M	74	neg	pos	0.328
6.5	2046	0.376	M	82	pos	pos	0.308
6.3	269	0.503	M	84	pos	pos	0.295
6.3	437	0.208	F	71	weak	pos	0.311
6.3	2095	0.344	M	65	pos	pos	0.302
6.3	2186	0.154	M	43	neg	neg	0.309
6.2	96	0.256	M	60	pos	pos	0.331
6.0	36	0.071	M	74	neg	neg	0.282
6.0	1373	0.136	M	82	pos	pos	0.302
6.0	2055	0.227	M	50	neg	neg	0.288
6.0	2179	0.150	F	62	neg	neg	0.294
6.0	2272	0.438	M	47	pos	pos	0.300
5.9	586	0.341	F	70	neg	neg	0.296
5.9	2079	0.354	F	71	pos	pos	0.280
5.9	2092	0.466	M	49	pos	pos	0.279
5.9	2138	0.450	F	47	pos	pos	0.289
5.8	504	0.430	M	53	pos	pos	0.288
5.8	1114	0.249	F	71	pos	pos	0.294
5.8	2185	0.395	M	45	pos	pos	0.286
5.7	20	0.427	M	66	pos	pos	0.267
5.7	296	0.312	F	60	neg	neg	0.305
5.7	2054	0.228	M	34	neg	neg	0.273
5.6	391	0.219	M	51	pos	pos	0.279
5.6	485	0.221	M	68	neg	neg	0.278
5.6	2167	0.265	M	33	neg	neg	0.278
5.5	112	0.471	F	77	neg	neg	0.257
5.5	260	0.195	M	50	neg	neg	0.294
5.5	2160	0.348	M	57	pos	pos	0.272
5.4	473	0.234	M	56	pos	pos	0.266
5.4	2136	0.468	F	69	pos	pos	0.267
5.4	2137	0.455	M	77	pos	pos	0.264
5.3	383	0.109	F	87	weak	pos	0.264
5.3	494	0.212	M	57	neg	neg	0.260
5.3	1271	0.179	M	59	pos	pos	0.267
5.3	2006	0.246	M	34	neg	neg	0.268
5.3	2102	0.484	M	68	pos	pos	0.261
5.3	2143	0.223	F	68	neg	neg	0.260
5.2	362	0.110	M	66	neg	neg	0.282
5.2	2161	0.303	M	58	pos	neg	0.256
5.1	523	0.457	F	53	pos	pos	0.253
5.1	1378	0.327	M	70	neg	neg	0.258
5.1	2009	0.264	M	52	neg	neg	0.259
5.1	2101	0.501	F	68	neg	neg	0.245
5.1	2188	0.471	F	61	neg	neg	0.250
5.0	69	0.423	M	69	pos	pos	0.269
5.0	74	0.266	M	68	neg	neg	0.270
5.0	2148	0.207	F	41	neg	neg	0.246

p120 UNITS	Sample	ELISA	Sex	Age	Blot	Anti-CagA Ab	p120 ELISA
4.9	132	0.135	M	61	neg	neg	0.263
4.7	38	0.491	M	55	pos	weak	0.221
4.2	196	0.428	M	71	pos	weak	0.198
4.1	340	0.251	M	42	neg	neg	0.221
4.0	256	0.140	M	74	neg	neg	0.217
3.8	3	0.488	F	69	weak	neg	0.179
3.8	61	0.252	M	85	pos	neg	0.205
3.8	306	0.092	M	76	neg	neg	0.204
3.7	121	0.234	M	78	neg	neg	0.198
3.7	318	0.368	F	83	neg	neg	0.198
3.6	16	0.100	F	69	neg	neg	0.170
3.6	183	0.513	M	62	pos	weak	0.171
3.5	101	0.604	M	71	pos	neg	0.186
3.5	364	0.424	F	66	pos	neg	0.189
3.3	66	0.188	M	51	neg	neg	0.157
3.3	82	0.349	M	72	neg	neg	0.178
3.3	115	0.115	M	52	neg	neg	0.178
3.3	291	0.275	M	68	neg	neg	0.179
2.9	52	0.194	M	76	neg	neg	0.138
2.9	293	0.265	M	57	neg	neg	0.157
2.8	278	0.083	F	67	neg	neg	0.151
2.7	148	0.187	M	67	neg	neg	0.127
2.7	271	0.469	M	63	pos	neg	0.128
2.6	353	0.548	F	87	pos	neg	0.138
2.5	55	0.492	M	66	pos	neg	0.132
2.5	70	0.114	M	61	neg	neg	0.117
2.5	78	0.564	M	83	pos	neg	0.133
2.5	345	0.256	M	68	neg	neg	0.136
2.2	1	0.136	F	85	neg	neg	0.103
2.2	150	0.144	M	50	neg	neg	0.105
2.2	400	0.270	M	63	neg	neg	0.107
2.0	294	0.061	F	87	neg	neg	0.107
2.0	313	0.189	M	60	neg	neg	0.107
1.9	327	0.362	F	38	neg	neg	0.102
1.8	164	0.154	M	54	neg	neg	0.083
1.8	179	0.150	M	41	neg	neg	0.099
1.8	195	0.369	M	69	pos	weak	0.086
1.8	323	0.295	M	56	neg	neg	0.095
1.7	24	0.100	M	68	neg	neg	0.081
1.7	214	0.520	M	62	weak	neg	0.082
1.5	48	0.077	M	74	neg	neg	0.071
1.5	190	0.076	F	71	neg	neg	0.082
1.5	199	0.096	M	65	neg	neg	0.082
1.5	231	0.136	M	57	neg	neg	0.082
1.5	301	0.230	M	66	neg	neg	0.078
1.5	334	0.290	M	74	neg	neg	0.081
1.3	83	0.120	F	64	neg	neg	0.063
1.2	76	0.106	M	65	neg	neg	0.062
1.2	167	0.171	F	69	neg	neg	0.056
1.2	223	0.173	M	55	neg	neg	0.064
1.1	46	0.360	M	61	weak	neg	0.054
1.1	77	0.130	M	60	neg	neg	0.060
1.1	181	0.381	M	42	pos	weak	0.050
1.1	343	0.289	M	60	neg	neg	0.057
1.0	75	0.113	F	62	neg	neg	0.049
1.0	135	0.141	F	68	neg	neg	0.045
1.0	176	0.100	M	55	neg	neg	0.053
1.0	227	0.234	F	72	neg	neg	0.056
1.0	350	0.327	M	54	neg	neg	0.055
0.9	32	0.255	M	72	neg	neg	0.044
0.9	275	0.114	M	68	neg	neg	0.050
0.8	81	0.152	F	67	neg	neg	0.036

p120 UNITS	Sample	ELISA	Sex	Age	Blot	Anti-CagA Ab	p120 ELISA
0.8	289	0.197	M	80	neg	neg	0.043
0.6	155	0.080	M	69	neg	neg	0.026
0.5	145	0.112	F	83	neg	neg	0.024
0.4	33	0.396	M	69	neg	neg	0.020
0.4	111	0.081	F	34	neg	neg	0.021
0.3	151	0.067	M	64	neg	neg	0.013
0.2	100	0.085	F	69	neg	neg	0.010
0.2	236	0.187	M	47	neg	neg	0.011
0.1	60	0.103	M	60	neg	neg	0.005
0.0	35	0.117	M	74	neg	neg	0.000
0.0	142	0.087	F	84	neg	neg	0.000
0.0	171	0.178	M	67	neg	neg	0.000
4.5	31	0.197	M	42	pos	pos	0.212
4.1	261	0.352	M	82	pos	pos	0.194
3.3	189	0.092	F	75	weak	pos	0.177
3.0	26	0.406	F	68	weak	pos	0.141
2.9	389	0.318	M	60	pos	pos	0.142
2.5	173	0.068	M	73	weak	pos	0.134
2.5	354	0.450	M	62	pos	pos	0.134
2.2	13	0.231	M	64	weak	pos	0.105
2.1	372	0.378	M	64	pos	pos	0.105
2.1	385	0.326	M	71	pos	pos	0.102
1.9	85	0.577	M	60	pos	pos	0.103
1.8	395	0.366	M	64	pos	pos	0.088
1.6	320	0.523	M	72	pos	pos	0.084
1.2	303	0.323	F	67	weak	pos	0.064

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