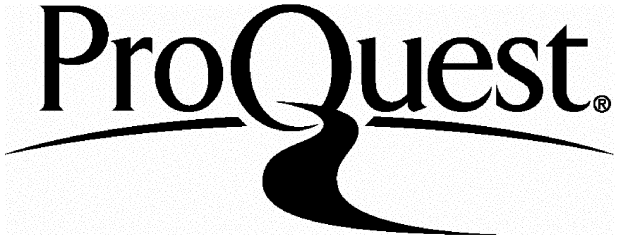
THE DIRECTION OF RELATIONSHIP BETWEEN *HELICOBACTER PYLORI* AND DUODENAL ULCER.



THE DIRECTION OF RELATIONSHIP BETWEEN *HELICOBACTER PYLORI* AND DUODENAL ULCER.

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by

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ABSTRACT

Prevalence of *H pylori* was determined in 208 patients undergoing endoscopy for dyspepsia. Results were expressed separately for methods involving and not involving polymerase chain reaction (PCR). The results confirmed well-established findings such as a greater prevalence in duodenal ulcer patients than in groups with other diagnoses, and increasing prevalence with age.

The unselected records of 320 different patients undergoing endoscopy for dyspepsia were studied using logistic regression analysis to determine factors significantly associated with a) DU and b) *H pylori.* It was found that number of endoscopies was significant for both regressions. In order to avoid the compounding effects of repeated endoscopies, a comparison was made in patients undergoing endoscopy for the first time in the series between those who had and those who had not undergone a previous endoscopy. This finding indicated that the relationship between prevalence and number of endoscopy was spurious. A search for the explanation revealed that the number of endoscopy was related to the duration of duodenal ulcer symptoms. Applying this information, it was found that 5 patients with symptom of <6 months were free of the infection, but the prevalence after 6 months was 96%. It is concluded that *H pylori* is most unlikely to be a prime factor in the aetiology of duodenal ulcer.

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My thanks are also due the many patients who participated in this study, in the full knowledge that often it is as much for my benefit as theirs!

Finally, I am grateful for the grant support of the Stanley Thomas Johnson Foundation, Switzerland.

DEDICATION

To Ronke, Damilola and Eni with love. The joys of sharing with you are boundless.

CHAPTER ONE
INTRODUCTION AND HISTORICAL REVIEW.

DECLARATION - **The series of investigations in this study, including data analysis and writing-up, were conducted between October 1995 and July 1998. All the clinical and technical laboratory work, with the exception of culture and histology, were carried out by myself.**

1.1 INTRODUCTION

Duodenal ulcer (DU) is a *multifactorial* disease, with an interplay of aggressive factors and protective mucosal mechanisms. Despite extensive research over many years, the aetiology of DU in man remains largely unknown. During the last century, the dictum was Schwartz's 'No acid; no ulcer'. Current opinion is that the infection of the stomach with *Helicobacter pylori* is the main cause. The chief reasons for this view are (a) that eradication of the infection with antibiotics and bismuth achieves a rapid and long-lasting remission; and (b) that many other studies have found a prevalence of *H pylori* in DU as high as 90-100% (Rauws et al 1988, Diomande et al 1991, Rohrbach et al 1993, Tytgat et al 1993, Lin et al 1993, Huang et al 1993, Sandikci et al 1993)

Previous studies from this department measured prevalence of *H pylori* in several groups of dyspeptic patients as well as in healthy control groups. In 1991, Chandrakumaran and Vaira found a low prevalence of *H pylori* in DU (70%) in dyspeptic patients attending a routine endoscopy clinic. This study has only been reported in thesis form: it was criticized because this prevalence rate was so much smaller than

those reported by other authorities and the suggestion was made that the low prevalence was due to inaccurate procedures for detecting *H pylori*. Tunio in 1994 studied asymptomatic volunteers with age and sex matching Chandrakumaran's dyspeptic subjects, using the non-invasive ^{13}C- urea breath test. This showed an overall prevalence of *H pylori* in asymptomatic controls of 39%, and compared well with reports from other western countries, but provided further confirmation that the infection could be present without producing an ulcer.
The starting point for this investigation, therefore, was to repeat Chandrakumaran's study (a) to determine whether with standard procedures (culture, histology, rapid urease test), Chandrakumaran's low figure for procedure was or was not confirmed; and (b) to explore whether the very sensitive PCR method for detecting the organism would raise the prevalence to the 90-100% level reported by many other centres.

The chief difficulties with *H pylori* being the cause of DU are that (1) the infection is present in a large proportion of individuals who do not have a DU: indeed, in some countries the prevalence approaches 90%; and that (2) a small proportion of patients with a DU are not infected with *H pylori*.

The mechanism whereby dyspeptic patients acquire *H pylori* infection is as yet unknown. During 1994, Tunio also studied by logistic regression analysis the factors associated with *H pylori* infection and with DU and showed that the most important correlates were respectively the number of endoscopies that the patient had undergone, and smoking. The implications of these results were unclear, because they suggested that endoscopy itself was a factor in producing infection with *H pylori*, and

that smoking was more important than the organism in producing a DU. Further information was therefore sought on these two aspects.

The first investigation was further to examine the role of endoscopy in the transmission of *H pylori* infection by increasing the number of observations made by Tunio (1994). Related to this, further investigations were done to check the endoscopy cleaning procedure to determine whether they achieved sterility and to investigate whether there was any change in *H pylori* status after endoscopy.
Secondly, as it was thought that the endoscope itself might be pushing down the organism from the oral site, the mouth and gastrointestinal tract were examined for the presence of this organism, and for the relationship, if any, between strains of the organism found at more than one site.

During the course of these studies, I have attempted to keep an open mind. I am aware that when A and B co-exist, it is a common error to allow ones prejudice to determine the direction of the causal link. It might appear consistent with previous information that A is causing B. However, the true situation might be that B is causing A, or indeed that both A and B are caused by a third, unsuspected factor, C.

1.2 History links bacterial infection with ulcers

(References in this section are quoted by BJ Rathbone and RV Heatley, 1992)

Before the discovery of *H. pylori* and the subsequent work in this area, research as long as a century ago linked microorganisms with peptic ulcer.

Bottcher, in 1874, reported the presence of spiral organisms in the stomachs of mammals. This same finding was also demonstrated by Bizzozero in 1893 as he found spirochaetes in the gastric glands and parietal cells. Extending this study to other mammals, Balfour demonstrated spiral bacteria in dogs and monkeys. Kreinitz and Luger, however, have been credited with reporting the first case of isolation of this organism from a human.

Interest in bacteria as a cause for ulceration then dwindled as some studies were unable to find the bacteria. Some cases of viral colonization were even reported.

When Luck and Seth described the presence of considerable urease activity in the stomach in 1924, another line of evidence, it seems, had been reached. This finding was heavily substantiated much later when, in 1959, Leiber and Lefevre showed that this urease might be bacterial in origin. This was based on the fact that the urease activity disappeared during administration of antibiotics. Much work still needed to be done to establish the connection between these various intriguing findings.

In 1975, Steer and co-workers reported spiral bacteria closely applied to the mucus-secreting cells in the deep layer of the stomach of gastric ulcer patients. He demonstrated that the mucus in these patients was

scanty compared to the mucus in normal stomach. This was unfortunately met with general disbelief as the culture only yielded *Pseudomonas aeruginosa,* regarded as a mere endoscopic contaminant!

1.3 The discovery of *H. pylori*

Interest in bacterial colonization of the stomach was rekindled in the 1970s with the advent of fibreoptic endoscopic techniques, which permitted biopsy of the antrum.

In 1979, Fung correlated endoscopic, histological and ultrastructural findings in chronic gastritis. Using light and electron microscopy, he showed that bacteria did not invade epithelial cells and concluded that they were not pathogenic. Despite this, several other researchers maintained their interests, using methods for culture as they became available.

The first culture of *Helicobacter pylori* was in Royal Perth Hospital, Western Australia, in the early 1980s. Barry Marshall, an internist at the hospital, in collaboration with Robin Warren, a histopathologist, worked on a research project investigating a bacterium known from earlier studies to be associated with gastric inflammation. Initial attempts to culture the organism from the gastric biopsies, using non-selective media for the usual period of 48 hours, produced no growth. The first successful culture occurred accidentally, when the 35th biopsy was incubated during the Easter holidays in April 1982 and in consequence was only examined after 5 days' incubation. A heavy

growth of bacteria occurred. When other biopsies were later incubated for 3-4 days a similar growth was seen. This organism was named *Campylobacter pylori* as it resembled the organism in the genus *Campylobacter* and was found in the region of the pylorus.

Independent reports from Rollason et al (1984) and Steer et al (1984) showed similar growth of the spiral organisms and the association with gastritis and peptic ulcer.

This new microorganism was first named *Campylobacter*-like organism (CLO) because of the similarity to the genus *Campylobacter.* It was later changed to *Campylobacter pyloridis* since it was mainly in the pyloric region of the stomach. This was grammatically corrected to *Campylobacter pylori.* However, biochemical and ultrastructural characteristics of this organism showed that the 16s ribosomal RNA did not have the characteristic sequence found in *Campylobacters.* It was therefore classified into a new genus, *Helicobacter* (Goodwin et al 1989)

1.3.1 **The genus *Helicobacter*.**

The advent of techniques of molecular biology has revealed other bacteria classified in the genus *Helicobacter.* Members of this genus are characterised by; sequence analysis of 16S rRNA; presence of one or more polar, sheathed flagella; their cellular fatty acids, and; biochemical tests such as that from the enzyme urease.

The ribosomal RNA molecule facilitates protein synthesis on the bacterial ribosome, an organelle that has many conserved regions despite evolutionary changes over the millennia. Thus, bacteria with sequences with more than 90% homology are closely related. So far, nine species of the genus have been identified and are listed in table 1.

It is apparent that *H. pylori* only colonizes the human stomach. These other bacteria that have been observed in the stomachs of animals, offer hope of animal models for the human disease in our understanding of *H. pylori* infection. Knowledge of these organisms in animal models may provide clues to how *H. pylori* survives in the stomach and how it can influence gastric physiology and contribute to gastric pathology.

Helicobacter felis

The origin of this spiral bacterium has been linked to cat, hence its name. The distinctive characteristic is its periplasmic fibrils. It has been reported to cause extensive neutrophil infiltration as seen with *H. pylori* gastritis. However, it is generally considered to be incapable of being transmitted to the human gastric mucosa, partly because of its very low chance of human ingestion.

Helicobacter mustelae

Fox et al in Boston first isolated this organism from the ferret. It has not been found to colonize any other animal. In the ferret stomach, it is located down the gastric pit rather than lying free. It does not have a spiral shape but a short rod-shaped bacterium. Its multiply sheathed

flagellum is unusually lateral, probably accounting for its very distinctive spinning mobility.

Helicobacter nemestrinae

This was isolated from the gastric mucosa of a *Macaca nemestrina* monkey. It is morphologically so close to *H. pylori* that it was at first thought to be a human strain transmitted to the monkeys by their human handlers!

Helicobacter acinonyx

Isolated from the gastric mucosa of cheetahs in Ohio.

Helicobacter heilmanii(Gastrospirillum hominis)

Light microscopy of this bacterium in the gastric mucus of dog showed the very tightly spiralled morphology which is its characteristic feature. It has more than 93% of the sequence of bases in the 16s ribosomal RNA molecule in common with *H. pylori*. It is 7 to 10 μm long with tufts of multiple flagella at both ends. It is widely distributed in animals such as cat, dog, monkey, baboon, pig, cheetah etc. Studies have shown that this organism is localized mainly near the fundic-pyloric junction and in the cardia, invading the gastric pits, gastric glands, parietal cells and stimulating large lymphoid nodules in the mucosal lamina propria. Perhaps this organism, like *H. pylori*, can infect the human. In a series of 1300 human biopsies, *Gastrospirillum hominis* was found in 3

patients. Numerous studies have associated this organism with gastritis and ulcers.

Helicobacter muridarum

Perhaps the first *Helicobacter* to be isolated. This initially lives in the small and large bowel of rodents, but as these mice age, the organism moves up into the stomach and causes a significant gastritis.

Helicobacter ciaedi and fennelliae

These are urease negative and are non-gastric. They have been isolated from the rectum of homosexuals with proctitis, proctocolitis or enteritis.

1.4 THE ORGANISM : *Helicobacter pylori*

1.4.1 The morphology of *H. Pylori*

This has been described in detail with the use of light and electron microscopy. It is a unipolar, multiflagellate, spiral organism with bluntly rounded ends. It measures 0.5 to 1.0 μm in width and 2.5 to 4.0 μm in length. The cell wall is covered with ringlike subunits 12 to 15 nm in diameter, with the characteristics of gram negative organisms.

The external surface is covered by glycocalyx which links up with epithelial surfaces in gastric mucosa.

The flagella are sheathed and are attached in a bunch of four to six to one pole of the organism, ending in a membranous terminal bulb. It is an obligate microaerophilic rod, with a gently spiral shape. When cultured on solid media, true spiral forms may be few or absent. Prolonged culture gives rise to the emergence of coccoidal forms, which also appear after exposure to oxygen. The coccoidal is believed to be a dormant state, to assist the survival of the organism in an environment where conditions are not favorable for growth.

1.4.2. **Pathogenicity of *H pylori***

Helicobacter pylori's unique feature is its ability to colonize the human stomach. Gastric acid kills most bacteria but gastric *Helicobacters* have evolved some special features which allow them to live in this niche. It also sets up a mucosal inflammatory reaction consisting of large numbers of polymorphornuclear cells. To fit its need, *H. pylori* has both modified bacterial structures as well as acquired new virulence factors. These factors play complex roles in several phases of the infection, enabling the organism to colonize, persist and induce lesions.

1.4.2.1. Colonizing factors

Sufficient numbers of *H. pylori* must survive the gastric acid barrier and colonize fluid or mucous layer. If the bacterium cannot colonize the gastric mucosa, then it cannot induce disease. This has been emphasised in an important series which concluded that DU is associated with increased *Helicobacter pylori* density and suggests that a threshold of infection density in the gastric antrum is necessary for ulcer formation (Khulusi et al 1995).

Ultra structural studies described the association between *Helicobacter pylori* and gastric and duodenum mucosa. A striking feature of *Helicobacter pylori* is its exclusive relation to cells derived from gastric-type mucosa. The physicochemical properties of the mucus overlying gastric type cells may be responsible for this phenomenon. *Helicobacter pylori* can be found in the mucus layer resting on the short microvilli of the epithelial cells and especially at intercellular junctions. The spiral shape is well adapted to motility in the viscous gastric mucous layer and is thought to promote rapid passage of *H. pylori* through the acid milieu of the gastric lumen and its penetration of the mucous layer before colonizing the neutral environment immediately overlying the gastric epithelium. It is also thought to be required for the maintenance of a bacterial reservoir within the gastric mucus. In a study using increasing concentrations of methylcellulose, *H. pylori* was shown to travel much greater distances than flagellated non-spiral organisms such as *E. coli* thus demonstrating the advantage of the spiral shape.

The presence of flagella is also crucial. Biochemical and molecular genetic analysis of flagellar filament have characterised a further component, the flagellar hook. Animal experiments showed that mutants

in the flgE genes coding for the hook protein did not express either flagellin or the hook protein and were non-motile.

The cell wall of *H. pylori* has lectin-like adhesin which allows it to bind selectively to mucus and epithelial cells. Several adhesin-receptor systems that mediate adhesion have been described. Targets for these adhesins are said to exist in the gastric mucus as glycoproteins and glycolipids. Blocking adhesins is considered a therapeutic possibility as the binding was inhibited by bismuth subcitrate, though omeprazole and cimetidine had no effect. It has been shown that *H. pylori* lectins attach to red cells from animal species, an action that can be blocked by mono-sialogangliosides (Emody et al 1988). A fibrillar adhesin has also been identified which attaches to carbohydrate moieties on the intestinal cells. This results in pedestal formation where the bacteria adhere. These attachment pedestals are tight and it has been found that actin polymerization occurs below these areas, causing loss of the brush border and the microvilli, resulting in localised cell damage (Smoot et al).

Once established in the stomach, the pathogenic effect of *H. pylori* is produced directly or indirectly, by competition with the host for nutrients, elaboration of biochemical agents and stimulation of mucosal and systemic antibody and inflammatory response.

Urease and Ammonia

Among the various enzymes produced by *H. pylori*, urease is perhaps the most important to the pathogenic process. We know that this organism lives predominantly in the mucous gel overlying the gastric

epithelium. This site represents a niche remote from the host's antibacterial defenses, specific immunity and non-specific removal by low pH and peristalsis. *H. pylori* survives in gastric acid by metabolising the urea present, thereby producing a cloud of ammonia which surrounds the organism and forms a micro-environment. The ammonia produced disturbs the ionic integrity of the gastric mucus, allowing back-diffusion of hydrogen ion towards the gastric mucosa and resulting in tissue damage. Experimentally, ammonia generated by urea and urease has been shown to cause epithelial damage in rats. Ammonia is toxic to mammalian cells because it combines with alphaketoglutarate in the tricarboxylic acid cycle (Kreb's cycle) to form glutamine, thus depleting this essential substrate. In an in-vitro study ammonia produced vacuolation of epithelial cells (Xu et al). Animal experiments have shown that urease-negative mutants were essentially unable to colonise gnotobiotic piglets (Eaton et al 1991 and Krakowka et al 1987). This is presumably as a result of their inability to produce ammonia.

However, the role of ammonia in inducing parietal cell failure and, hence hypochlorhydria, is not yet well established. It is thought that ammonium ions compete with hydrogen ion for the H^+ K^+ ATPase of the parietal cell membrane, thus leading to excretion of ammonium rather than H^+ from the parietal cells.

Other enzymes:

H. pylori produces the enzyme catalase which significantly impairs the action of antibacterials. Normally, the neutrophils generate superoxide which is converted to toxic radicals responsible for killing bacteria. It has been suggested that release of catalase may protect *H.pylori* from

toxic long-chain fatty acids and their metabolites. This is because the enzyme catalase breaks down hydrogen peroxide into water and oxygen, thus preventing the production of toxic radicals.

Other enzymes produced by *H. pylori* include phopholipase A2 and C, mucinases, glycosidases. These affect the integrity of the epithelial cell membrane and the normal gastric mucus. Phospholipase A2 of *H. pylori* removes a long-chain fatty acid group from the second carbon, while phospholipase C removes the phosphate group from the third carbon of the phospholipid in the epithelial cell membrane. This results in impairment of the integrity of the phospholipid bilayer membrane and loss of hydrophobicity. Another effect is the liberation of arachidonic acid from the membrane phospholid resulting in the production of leukotrienes and prostaglandins through the lipooxygenase and cyclooxygenase pathways. This triggers an inflammatory response of varying magnitude.
The normal gastric mucus consists of a protein backbone with numerous attached carbohydrate moieties. Incubation of *H. pylori* with mucus can result in a large component of degraded mucin. Other inflammatory mediators synthesised and released by immunocytes of the *H. pylori*-inflamed gastric mucosa include interferon, platelet-activating factor, tumour necrosis factor alpha.

The presence of *H. pylori* in the stomach incites an intense gastric mucosal immune response. Local humoral immune response leads to the production of antibodies, promoting killing of *H. pylori* by polymorphonuclear leukocytes. Typical immunoglobulin produced is the secretory IgA. Immunoglobulins IgM, IgG and IgE may also be present. The antibacterial activity of monocytes and lymphocytes is enhanced by secretory IgA which is also important in inactivation of *H. pylori* toxins.

If the secretory IgA-antigen complex at the mucosal surface activates the complement system either by the alternate or classical pathway, a local inflammatory response would result. However, in the tissue, IgG coating of bacteria may activate the complement system directly, resulting in tissue damage.

Cellular immunity response has also been observed involving numerous lymphocytes in *H. pylori* -infected gastric mucosa. There is strong expression of class 11 antigens on gastric epithelial cells with HLA-DR being the most prominent.

Toxins:

Many strains of *H. pylori* produce a cytotoxin which induces acid vacuoles in eukaryotic cells by an autophagocytic mechanism (Figura et al 1989).This vacuolating toxin is an extracellular protein with a molecular weight of 87 kDa, encoded by the gene vacA (Luzzi et al). The vacuolation is inhibited by bafilomycin A1, a specific inhibitor of eukaryotic H^+ -adenosine triphosphatases (ATPases), suggesting that an ATPase proton pump is the target (Papini et al). Toxins which induce cell death are encoded by the gene cagA (cytotoxin-associated gene), which is a 128 kDa surface protein. It is believed that more than one toxic activity is expressed from the cagA gene. Immune responses to cagA are associated with atrophic gastritis, duodenal ulcer and gastric cancer. The cagA and vacA proteins are present in only 70% of *H. pylori* strains. This may explain why not all *H. pylori*- infected individuals develop ulcer disease.
However, the genetic subtype of cagA have been further analysed. *H pylori* strains that possess the cagA (i.e cagA-positive) are associated

with increased levels of inflammation and therefore, increased risk of more severe gastric diseases, including peptic ulcer, atrophic gastritis and gastric cancer, than those without cagA (i.e cagA-negative), (Webb et al).

1.4.3 **Diagnosis of *H pylori***

There are several methods available for making a diagnosis of *H. pylori* infection. Each test has a unique feature which offers additional information to facilitate diagnosis, but none is perfect. When a precise result is expected, tests are usually combined. These tests are either invasive or non-invasive. The invasive method involves the use of an endoscope in obtaining biopsy specimens for culture, histology, urease test and polymerase chain reaction; or gastric juice is collected for the measurement of urea and ammonia concentration. The non-invasive method include urea breath tests and serology for *H. pylori* antibody.

1.4.3.1. Invasive method:

This is the obvious choice for a symptomatic patient. At the endoscopy, a clear assessment of the underlying pathology can be made. Also, a biopsy can be taken for further laboratory assessment, and culture and sensitivity can be done for those patients who require antibiotic therapy. However, this method has two potential sources of error:
Sampling error:

The endoscopic appearances in *H. pylori* infection are non-specific and by no means diagnostic. There is an uneven, patchy distribution of the

organism in the stomach. The bacterial growth is generally less if there is mucosal atrophy and intestinal metaplasia. By testing multiple biopsies, sampling errors are diminished.

Effect of therapy:

Drugs such as proton pump inhibitors and antibiotics, can greatly decrease the number of *H. pylori* in the stomach and produce a false negative result. These should be stopped prior to testing.

1.4.3.1.1. The biopsy urease test:

The basis of the urease test is the ability of *H. pylori* to produce the enzyme urease. Urease digests urea to produce carbon dioxide and ammonia, and the resultant ammonium ions increase the pH of the surrounding medium. This change can be detected with a pH indicator such as phenol red. A positive result is obtained when the pH indicator changes from yellow at pH 6.8 to red at pH 8.4.

The first urease test described was the Christensen's 2% urea broth; a number of variations of this have now been developed commercially.

The CLOtest developed by Barry Marshall, working with his father who is a chemist, is one of these modifications. It consists of Christensen's agar and an indicator mounted on a slide. The biopsy is pushed right into the gel and the kit is re-sealed and kept warm. Positive results are evident when the gel changes from yellow to pink, often within minutes

of addition of the tissue to the medium. False positive results may occur with lowered gastric pH as occurs in atrophic gastritis or drug therapy, when several other bacteria such as *Proteus, Klebsiella* species, can produce small amounts of urease. False negative results are usually due to sampling error or drug therapy. This test is cheap, requires no specific skills to carry out, and can provide a result before the patient leaves the clinic.

1.4.3.1.2. Histology:

Histology is a reliable method for detection of *H pylori* infection and it also gives information on the state of the mucosa. The bacterium is identifiable by virtue of its characteristic mophorlogy and its preferential distribution in the stomach.

When first identified, special stains were needed. Warren and Marshall used the Warthin-Starry silver stain which makes the organism appear larger in the sections. However, this method is expensive, technically demanding and time consuming. Routine stains such as Giemsa and haematoxylin and eosin are now used by most histopathologists. Technically, these stains do not prove the organism seen to be *H pylori*; proof can only be obtained using specific immunostaining to differentiate similar-looking organisms such as *'Gastrospirillum hominis'*.

The specificity and sensitivity of this method compared to the culture and urease tests are both in the range of 95%. This depends on the expertise of the histopathologist. Organisms tend to be harder to detect

in surgical excision specimens where other bacteria are likely to be encountered than in biopsy samples. In these circumstances, specific immunostaining methods of identification may be desirable. False-negatives are likely to be due to antibacterial therapy and sampling errors. At least two biopsies within 5 cm of the pylorus should be taken to improve the chance of a correct diagnosis.

1.4.3.1.3. Culture:

The process of culturing *H pylori* is probably the most difficult approach to the diagnosis. The success depends on local technique and access to facilities. Because the growth is slow and requires anaerobic conditions, incubating for 96 hours or more in a moist microaerophilic atmosphere at 37 ^{0}C enhances the chance of culturing *H pylori.*

Transport::

Blood and chocolate agar are commonly used transport media. For a short period (3 hours), saline is quite suitable but not after 24 hours. It is crucial to use the appropriate transport medium for the time period in order to maintain viability.

Growth:

High humidity, carbon dioxide and pH 5.5 are optimal conditions for the growth of *H pylori*, optimal growth being at PO_2 of 2-10 Kpa, but not at the PO_2 of air. Under aerobic conditions, *H pylori* strains will grow with reduced viable cell counts and smaller colonies.

Identification:

Once cultured, the identification of *H pylori* is based on colony morphology, biochemical and immunological properties of the organism. Polymerase chain reaction (PCR) using primers derived from the urease gene, and immunological techniques with polyclonal and monoclonal antibodies directed against a 30 kDa protein of *H pylori* outer membrane proteins, are in use.

Antimicrobial susceptibility testing:

An important application for culture in selected settings is the determination of the antibiotic susceptibility profile of treatment-resistant organisms. Using the disc-diffusion test or the plate-diffusion method, minimum inhibitory concentrations can be determined for a range of antibiotic concentrations.

Typing:

Successful identification of *H pylori* strains depends on accurate typing because of species heterogeneity. PCR-based techniques are now increasingly used because of their rapidity and convenience.

1.4.3.1.4. Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) is a process by which a specific sequence of DNA can be selected and amplified in vitro. This newly developed technique is simple to perform as it is partly automated. Results could be available on the same day the specimen arrives in the laboratory.

Mullis and Faloona at Cetus corporation in Emery Ville invented this method in 1987. Since then it has been widely applied and modified for a variety of molecular studies. The original technique was applied by a group of workers in the Human Genetic Department at Cetus for the amplification of human beta-globin DNA and to the prenatal diagnosis of sickle cell anaemia (Saiki et al 1985, 1986; Embury et al 1987). PCR is highly versatile and can be used for a variety of purposes. It can detect extremely small amounts of target nucleic acid. The sensitivity, simplicity and speed as well as universal applicability reflect the explosive growth of its use in clinical, forensic, archaeological and molecular biology research laboratories.

The principle involved can be summarised as follows:
1. Amplification of specific DNA sequences from as few as 25 base pairs to 10,000 base pairs in length from the entire genome (Saiki et al 1988)

2. PCR is several orders of magnitude more sensitive than direct hybridization and requires only a single target DNA molecule that need not be highly purified.
3. Introduction of heat-stable Taq DNA polymerase enzyme has made the technique simple and quick, allowing the multiplication of a single DNA sequence over a million times in only a couple of hours duration.

Perfomance of PCR:

The components needed for PCR to take place are as follows:
1.Template DNA following extraction.
2.Two single-stranded oligonucleotide primers which are complementary to known sequences of target DNA.
3. Four deoxyribonucleotide triphosphates- dATP, dCTP, dGTP, dTTP
4. Taq DNA polymerase.
5. PCR buffer.
6. AmpErase Uracil N-Glycosylase (UNG)

Template:

The target of amplification by PCR can be either DNA or RNA. To amplify an RNA sequence the first primer-directed DNA synthesis reaction is performed with RNA-dependent reverse transcriptase. The second strand of complementary DNA is synthesised by adding a DNA dependent polymerase. (Chelly et al 1988, Hart et al 1988.)
RNA amplification has the advantage that the sequences sought are already present in large numbers and are therefore easy to detect.
The target DNA for PCR does not need to be particularly pure. It can be used straight from clinical, laboratory or archaeological specimens. Denatured DNA is suitable for analysis so long as the sequence to be

amplified remains intact. The ability to propagate crude DNA from amounts too minute for standard amplification (i.e. cloning) gives the method such extraordinary power and sensitivity that the DNA in fixed pathological specimens, buccal cells from mouth washes, human hairs, a single lymphoid, or sperm cells, or ancient mummies, can now be analysed. Moreover, in studies of infection using clinical specimens, the target sequence does not necessarily need to be from an actively replicating organisms since PCR can also detect latent forms (the DNA will still be present).

Primer:

The specificity of PCR amplification is based on two oligonucleotide primers that flank the DNA segment to be amplified with their 5' ends, and hybridize to opposite strands. Two single stranded oligonucleotides, complementary to known sequences of target template DNA, serve to amplify a targeted base pair fragment.

6- Oligonucleotides used for priming the polymerase chain reaction should be of at least 16 nucleotides and preferably 20-24 nucleotides in length. Primers should not have regions of homology between them to avoid extraneous primer products arising from primer-primer self- or cross- homologies during amplification. Primers that have about 50% G + C content function most satisfactorily. To make oligonucleotides that serve as primers, enough knowledge of the sequence of the target DNA, at least at the site of annealing, is required. Synthesis of the oligonucleotide primers itself is an automated procedure and simple to perform.

Oligonucleotides are used in most cases at a concentration of 1 μM in polymerase chain reactions and is usually sufficient for as many as 30 cycles. Higher concentrations can cause amplification of undesirable non-target sequences while sub-optimal concentrations lead to inefficient results.

Taq DNA polymerase:

PCR requires repeated thermal denaturation of the template DNA at high temperatures and for a number of cycles. Early applications of the PCR used *E. coli* DNA polymerase I, Klenow fragment (POLI- kf). With this template, it was necessary to replace the enzyme after each denaturation step as it was not thermostable.

A thermophilic eubacterial micro-organism capable of growth at 70-75 ^{0}C was isolated from a hot spring in Yellowstone National Park and first described by Brock and Freeze in 1969. From this micro-organism, *T.aquaticus* (strain YT1), a thermostable DNA polymerase, Taq DNA polymerase, was purified by Chien et al (1976). Taq polymerase is not denatured irreversibly by exposure to high temperatures (>90 ^{0}C). It is shown to retain 65% activity after a 50 cycle PCR when the upper limit temperature is 95 ^{0}C for 20 seconds in each cycle 15. This characteristic of Taq polymerase has obviated the need to add more polymerase after every cycle of PCR and further simplified the method leading to the automation of PCR by a variety of simple temperature cycling devices. In addition, the specificity of the amplification reaction is increased by allowing the use of higher temperatures for primer annealing and extension.

Taq DNA polymerase carries a 5'- 3' polymerization-dependent exonuclease activity but lacks 3'-5'exonuclease activity. Commercially two forms of Taq DNA polymerase are available. One is the native enzyme purified from *Thermus aquaticus*, the other is Ampli taq which is genetically engineered in *E. coli.* 1-2 units of Taq DNA polymerase are usually enough for a typical PCR reaction: excess enzyme may lead to non-target amplification.

Deoxyribonucleotide triphosphates (dNTP):

The optimal concentration of the deoxynucleotide triphosphates (dATP, dCTP, dGTP and dTTP) need to be determined for a successful PCR reaction. Their equimolar concentration is usually at 50-200 μM. At this concentration there is sufficient precursor to synthesize approximately 6.5 and 25 μg of DNA respectively. Higher concentrations may tend to promote misincorporation by the polymerase.

The amount of dNTPs present in a reaction will determine the amount of free magnesium available since dNTPs appear quantitatively to bind Mg^{2+}. Therefore, a change in the concentration of dNTP will significantly affect the reaction mixture if $MgCl_2$ is not adjusted appropriately.

Similarly, a stock solution of dNTPs (50 mM) should be adjusted to pH 7.0 with 1N NaOH so that the pH will not be below 7.1 during the preparation of the reaction mixture.

PCR buffer:

PCR buffer provides preferred pH and ionic strength for PCR reactions. The solution is made up of the following;

50 mM KCl

10 mM Tris.Cl (pH 8.3 at room temp.)

1.5 mM $MgCl_2$.

The concentration of $MgCl_2$ is highly critical and generally excess Mg^{2+} will result in the accumulation of non-specific amplification products and insufficient will reduce the yield. The concentration of magnesium ion required to achieve optimal PCR amplification is dependent on the specific set of primers and template used.

Mg^{2+} is also superior to other divalent cations such as Mn^{2+} or Ca^{2+} which are altogether inefficient.

AmpErase UNG (Carry-over prevention):

Exogenous templates can lead to false positive amplifications especially products from previous PCR amplifications. This is possible because of the high degree of sensitivity of PCR. The AmpErase UNG has been recently developed to provide a means for ensuring that the products of

previous PCR amplifications cannot be reamplified to produce false positive results.

This enzyme can be encoded by the *E. coli* Uracil N-glycosylase gene which has been inserted into an *E. coli* host to direct the expression of the native form of the enzyme.
The mechanism involves enzymatic and chemical reactions analogous to the restriction-modification and excision-repair systems of cells to degrade specifically PCR products from PCR amplifications or to degrade mis-primed, non-specific products produced prior to specific amplifications, but not to degrade native nucleic acid templates.

CHAPTER TWO
CLINICAL IMPLICATION OF *H PYLORI*

2. CLINICAL IMPLICATION OF *H PYLORI* INFECTION

2.1 Peptic ulcer and *H pylori* infection.

Peptic ulcers are defects in the integrity of the mucosa of the upper gastrointestinal tract, that penetrate through the muscularis mucosae. In the evolution of peptic ulcer, it has long been thought to have a multifactorial pathogenesis, with an interplay between aggravating and relieving factors. Peptic ulceration is a commonly occurring disorder. The point prevalence of peptic ulcer is between 1 and 2%, whereas the lifetime prevalence of duodenal ulcer is 12%. It has a high relapse rate and is associated with significant morbidity and mortality arising mainly from complications of bleeding and perforation.

A variety of host and environmental factors may determine the response to *H. pylori* infection and ulcer formation. The antrum is the predominant site of inflammation in patients with DU, acid secretion is maintained since the body and fundus are not affected, and this may protect the proximal stomach from more intensive bacterial colonization. Previously, the use of aspirin and other non-steroidal anti-inflammatory drugs as well as smoking, stress and increased pepsinogen production were considered to be risk factors in the pathogenesis of peptic ulcer disease.

Genetic susceptibility to DU is increased in individuals with blood group O (cell attachment of *H. pylori* is facilitated by Lewis blood group antigens which are expressed more frequently in blood group O) and non- secretor status, and to GU in those with blood group A. Men are

more prone to ulcers than women, yet there is no significant difference in the prevalence of *Helicobacter* between the sexes. DU are also associated with the HLA DQ1 and HLA B5 phenotypes, and the rare autosomal dominant disease, hyperpepsinogenaemia. Irritants such as NSAIDS, aspirin, corticosteroids and cigarette act in synergy with *Helicobacter* to cause chronic inflammation. NSAIDS can lead to ulcers in the absence of *H. pylori* infection, while no duodenal ulcer recurred in smokers or non-smokers during a mean follow-up period of 3 years after *Helicobacter* eradication..(Mohammad et al 1994).

The discovery of *H. pylori* has made an enormous impact on the treatment and course of the disease. *Helicobacter pylori* is now thought to play an aetiological role in the pathogenesis of most duodenal ulcers and non-malignant, non-drug- related gastric ulcers.

In gastric ulcer patients, gastritis is a primary phenomenon and this precedes the ulcer. Patients with GU are more likely to have associated pangastritis, atrophic changes, intestinal metaplasia and lower acid secretion. These conditions lead to a more diffuse spread of the bacteria throughout the stomach and to the lowering of colonization densities, particularly in the antrum. (Fiocca et al 1992, Louw et al 1993).
Both duodenal and gastric ulcer disease are closely associated *with H. pylori* infection. *H. pylori* infection can be diagnosed in 90-100% of duodenal ulcer patients and in 60-100% of gastric ulcer patients (see tables 2 and 3). The estimated lifetime risk for peptic ulcer disease in *H. pylori*-infected subjects is approximately 15%. (Kuipers et al 1995, Valle et al 1996) which is at least 3-4 fold higher than in non-infected subjects. This risk appears to be dependent upon the amount of inflammation that is caused by the colonizing *H. pylori* strain. (Miehlke et al 1995)

The evidence for this association is based on correlation between duodenal ulcer disease and gastric metaplasia with *H. pylori* colonization in the duodenal bulb. There is also complete resolution of inflammation of the mucosa within 6-12 months of eradication therapy and the absence of ulcer relapse in patients from whom *H. pylori* has been eradicated. Eradication has been shown to have a major impact on the clinical course of both gastric and duodenal ulcer disease. (Hopkins et al 1996)

Eradication is said to lead to cure of the peptic ulcer disease and long term remission. A recent meta-analysis including data from 700 patients showed that the 12 month recurrence rate in patients in whom *H. pylori* was not eradicated was 80% compared with only 4% in patients in whom eradication was successful. But re-infection after eradication occurs at similar rate to the infection rate, and there is no protection from re-infection after the cure of a first infection. This, however may be due to recrudescence of low grade infection rather than a true re-infection. (Marshall et al 1994)

Further evidence for the role of *H. pylori* in peptic ulcer disease comes from observations that only certain strains cause ulcers in the presence of strain specific factors such as vac A and cag A. 80% of patients with DU were infected with cag A-positive strains of *H. pylori* and high titres of anti-cag A antibodies were found in the serum.

On the other hand, it is not totally convincing yet that *H. pylori* causes peptic ulceration in humans; duodenal ulcers have been absent in human volunteers and animals infected with *H pylori.* (Barthel et al 1988, Gregson et al 1989, Dooley et al 1989, Krakowa 1987, Lee 1990)

It is not clear why some people develop peptic ulcer while others infected with *H pylori* do not. Koch's postulate of a causal link between a disease and bacteria suggested that the organism must be found only in subjects with the disease. *H pylori* has been found not only in duodenal and gastric ulcers, but also in NUD (Veldhuyzen-van-Zanten et al 1990) and even in 40-100% of asymptomatic subjects (Koch et al 1990) and in entirely symptom-free subjects (Majmudar et al 1990, Dehesa et al 1991). However, in the light of our present knowledge of infection, there are some diseases where Koch's postulates are never satisfied and therefore, may not entirely be appropriate. Examples of these include *C defficile* and pseudomembranous enterocolitis; meningitis and *meningococcus* and *pneumococcus* organisms.

Although many authorities are convinced that *H pylori* is an important causal factor in DU formation, there is no direct evidence in humans that *H pylori* precedes the development of DU. Such evidence against a causal role for *H pylori* in DU is further substantiated by the high prevalence of *H pylori* in some areas which is not matched with an increased incidence of peptic ulcer. For example, epidemiological studies from Nigeria which has a very low incidence of peptic ulcer (32/1000), found that the majority of the population were positive for *H pylori* antibodies (85%) (Holcombe et al 1992a and b). Similar studies from the Far East have suggested a low prevalence of *H pylori* in patients with DU. Uyub et al 1994 found a low prevalence of *H pylori* in DU subjects(53%); Hsu et al 1992 found 76% of DU were positive for *H pylori* compared to 27% in NUD. Similarly, Kachintorn et al 1992 found *H pylori* in 66% of DU subjects.

Studies among different ethnic groups in Fiji also showed variation; native Fijians have a low incidence of DU compared to Indian-Fijians,

but there is no difference in prevalence of *H pylori* infection (Parshu et al1975, Beg et al 1988)

Helicobacter pylori infection predominantly involves the antral mucosa of the stomach. However, the ulcers most closely associated with the infection occur not in the gastric antrum but in the duodenum. This suggests that the mechanism by which the infection predisposes the patient to ulceration is not merely by directly damaging the mucosa, but may have two components.

H pylori plays a direct role in causing the mucosal damage in the duodenum which may lead to ulceration. If *H. pylori* is to have a direct role in duodenal mucosal damage, colonization of the duodenal mucosa is necessary (Johnson et al 1986). Studies have shown that *H pylori* is found in the duodenum in patients with duodenitis and duodenal ulcer, showing active mucosal inflammation, with areas of gastric type epithelium. (Steer 1984)

However, *H pylori* is unable to colonize small intestinal epithelium, as demonstrated by the sparing of intestinal metaplasia in chronic gastritis. In the same way, the distribution of *H pylori* in the duodenum is restricted to foci of gastric epithelium in the duodenal mucosa. (Thomas 1984, Wyatt et al 1988)

The mucous barrier is thought to be disrupted directly by *H pylori* through the following mechanisms:-
(a) production of ammonia, cytotoxin and phospholipase.
(b) enhancement of aggressive factors such as acid, pepsin, platelet activating factor.

(c) activation of monocytes and macrophages with release of tumour necrosis factor, interleukin-1 and reactive oxygen metabolites.
(d) production of antibodies directed against the organism that also react with gastric tissue (autoantibodies).
H. pylori gastritis is associated with the pathophysiological disturbances present in duodenal ulcer patients. The pathophysiological disturbance as a result of the inflammation causes a disruption of the inhibitory control of gastrin release. This is more marked if the infecting organism is a cag A-positive strain. (McColl et al 1991)

The gastrin link hypothesis is the most important of the mechanisms by which *H pylori* causes DU. Gastrin, a polypeptide secreted by antral G cells, stimulates parietal cells to secrete acid and, to a lesser extent, chief cells to secrete pepsin. Gastrin secretion is an important factor in the release of gastric acid into the stomach. (Wolfe et al 1988). It has been shown that individuals with DU and antral *H pylori* infection had significantly higher basal and meal-stimulated plasma gastrin concentration and higher peak, but not basal, acid output than individuals with DU who are not infected with *H pylori* (Levi et al 1989a and b).

Ammonia produced by hydrolysis of urea catalysed by urease from *H pylori* is thought to increase the pH of the mucus layer overlying the gastric mucosa. This interferes with the normal feedback inhibition of gastrin by luminal acid, resulting in a raised gastrin secretion. Gastrin either directly stimulate the parietal cells or indirectly exerts a trophic effect on parietal cell mass, or both, leading to excessive gastric acid secretion. The amount of acid that is overproduced in response to this increase in gastrin depends on the sensitivity to gastrin, which appears to be largely a host, and possibly a genetic, factor. Progressive damage to

the duodenal mucosa leads to the development of gastric metaplasia as a protective response. But the infection is able to colonize this area leading to further mucosa damage and subsequently, the development of duodenal ulcer disease.

Increased basal and stimulated acid output by the stomach body was recognized as a characteristic of and related to duodenal ulcer disease before *H pylori* infection was discovered. Schwartz's dictum of 1910, "no acid, no ulcer" has held true. It has been consistently demonstrated that basal and meal-stimulated gastrin levels are elevated in *H pylori* infection. However, corresponding consistent basal and peak acid outputs have not been demonstrated. (Levi et al 1989a, Graham et al 1991)

In one study, eradication of *H pylori* decreased basal and meal-stimulated gastrin secretion but not basal and peak acid secretion. (Graham et al1990). In another very interesting study by El-Omar et al, healthy infected subjects and infected ulcer patients had a similar degree of increase in gastrin secretion, while ulcer patients had a much more marked rise in acid secretion. Four groups of patients were studied: *H pylori*-negative healthy volunteers; *H pylori*-positive healthy volunteers; *H pylori*-positive patients with DU; and the same patients 1 year after eradication therapy. In response to gastrin-releasing peptide, there was a similarly exaggerated gastrin response in the infected healthy volunteers and in the patients with DU. However, the acid response to gastrin-releasing peptide was more marked in the DU patients than infected healthy volunteers, being sixfold in the former and threefold in the latter compared to *H pylori*-negative healthy volunteers. This increased acid response was largely resolved following eradication of the infection.

Other authors have claimed that hypergastrinaemia in DU is largely related to *H pylori* infection, but the increase in acid output was not. (Peterson et al 1993). The discrepancy may also be due to failure to make corrections for pyloric loss, duodenogastric reflux and stature. (Chandrakumaran et al 1994). Others have shown that chronic *H pylori* infection does not increase maximally stimulated acid output since there is gastric atrophy and consequently, reduction in the parietal cell mass. (Calam 1993)

Gastrin circulates in the systemic circulation and stimulates the parietal cells to secrete acid. When acid builds up in the antrum of the stomach, it exerts a negative inhibitory control to prevent further acid secretion which might damage the stomach and duodenum. Thus, as the pH in the antrum falls, it stimulates the release of stomatostatin from somatostatin-producing (D) cells. This important inhibitory control mechanism is thought to be disrupted by *H. pylori*, causing excessive acid secretion. Moss et al have shown that somatostatin mRNA levels were low in the presence of *H pylori* infection and rose significantly after treatment. In contrast, gastrin mRNA levels showed little variation between pre- and post-treatment levels. (Moss et al 1992).

Similar results have shown that in the presence of infection, somatostatin levels in the antral mucosa were significantly reduced but there was relatively little change in gastrin levels. (Queiroz et al 1993). The mechanism by which *H pylori* infection results in depletion of antral somatostatin may be related to elevation of antral surface pH by ammonia produced by bacterial urease activity. This local ammonia may block the acid-mediated trophic effect on D cells. Alternatively, a biosynthetic amine, N-alpha-methyl histamine, produced by *H. pylori*

may suppress somatostatin synthesis by interfering with the histamine-3 receptor (Courillon-Mallet et al 1995).

Histamine has been established as a potent stimulus for gastric secretion. Infusion of histamine in-vivo and in-vitro results in high acid production. This mechanism has also been linked with *H. pylori* infection. When the histamine concentration in oxyntic mucosa in patients infected with *H pylori* was measured before and after treatment, there was an increase in histamine concentration after the eradication of *H pylori*. (Queiroz et al 1993)

In summary, the presently accepted view is that in ulcer patients *H pylori* infection occurs predominantly in the antral region of the stomach. The accompanying inflammation disrupts the inhibitory control of gastrin release, and this is more marked if the infecting organism is a cag A-positive strain. This, in addition to the local mucosal damage by the bacterium itself, leads to the development of peptic ulcer disease.

Peptic ulcer complications:

The introduction of H_2-receptor antagonists and subsequently of the proton pump inhibitors has markedly diminished the incidence of complications in peptic ulcer disease (Bardhan et al 1989, McConell et al 1989). At present the majority of surgical procedures are emergency interventions for complications like haemorrhage, obstruction or perforation (Svanes et al 1993). These complications occur in 5-10% of duodenal ulcers. Perforated peptic ulcer disease is associated with

Helicobacter pylori infection, but the proportion of DU presenting as perforations is lower than the average DU- *H pylori* association (Sebastian et al 1995). In uncomplicated peptic ulcer disease, less than 10% of the successfully treated patients with *H pylori* infection and DU have a relapse (Moss et al 1992, Marshall 1994).
Thus, it has been suggested that anti-*H pylori* treatment following surgical treatment would prevent ulcer recurrence and that primary definitive surgery, such as highly selective vagotomy, is no longer justified in the treatment of complicated peptic ulcers (Greve et al 1997).

2.2 Gastritis and *H pylori*

Acute infection of the stomach with *H pylori* can be entirely symptom-free and pass undetected but some patients develop a clinical syndrome of epigastric colic, nausea, vomiting, flatulence, malaise etc., persisting for about 1-2 weeks (Marshall 1986). The natural history of *H pylori*-induced gastritis is not uniform and there are various levels of genetic predisposition mediated through mucosal defense and immunological mechanisms. First infection causes a severe pangastritis. Patients in whom the infection persists and becomes restricted to the gastric antrum are most likely to develop duodenal ulcer disease, while marked chronic pangastritis predisposes to gastric ulcer and gastric cancer. Progression from acute neutrophilic gastritis to chronic gastritis is a frequent event but ingestion studies have also shown that spontaneous clearance of

infection with resolution of the inflammatory reaction is possible (Morris et al 1987).

In early *H pylori*-gastritis, the first changes appear to be a neutrophil polymorph infiltrate of the mucosa and these cells may also exude from the epithelial cell surfaces. This form contrasts with the more frequently seen acute haemorrhagic gastritis found with NSAID use or alcohol injury, where there is epithelial exfoliation, oedema and haemorrhage into the lamina propria but little or no polymorph response. *H pylori*-gastritis is also non-specific because the histology, which commonly includes eosinophilic infitrates and granulomas does not predict the cause or the associated clinical condition.

The commonest form of chronic gastritis worldwide is caused by *H pylori*. In chronic gastritis, which occurs after 1-2 weeks, the cellular infiltrate alters to one predominantly of lymphocytes and plasma cells. Active chronic gastritis is diagnosed when intraepithelial and interstitial neutrophil polymorphs are present, in addition to lymphocytes and plasma cells. *H. pylori* positivity ranges from 70 -100% in active chronic gastritis. Focal collection of lymphoid follicles are frequently seen when the inflammatory cell infiltrate extends through the full thickness of the mucosa, especially during infection in children, and may be identified endoscopically. Progression to diffuse antral and corporal atrophy is also seen, which is recognized by glandular loss and separation of the remaining glands by fibrous tissue. The release of proteases by polymorphs and the production of other cytotoxic factors in the inflammatory and immune response could lead to this glandular destruction. This is thought to be provoked by *H pylori* infection (et al 1990).

Epidemiological studies have shown a pattern of acquisition of *H pylori* with age similar to that of gastritis. Serological tests for *H pylori* infection show that *H pylori* infection is low in children, but rises dramatically in the fifth and subsequent decades, and that more than half of the population over 50 years is infected. This seems to be due to a continous risk of infection (Veldhuyzen van Zanten et al 1994).
Although *H pylori* infection is low in children, acquisition of the infection may not be entirely a matter of aging. It has been suggested that close person to person contact is an important determinant of seroprevalence of *H pylori* in adulthood, indicating that the infection is transmitted directly from one person to another and may be commonly acquired in early life (Webb et al 1994).
Other evidence implicating *H pylori* in gastritis can be summarised as follows:-
(a) the voluntary ingestion of *H pylori* resulting in chronic gastritis
(b) antimicrobial therapy that clears infection clears gastritis
(c) *H pylori* only overlies gastric epithelium
(d) *H pylori* infection elicits a systemic immune response
(e) association of *H pylori* with epidemic gastritis and hypochlorhydria.

Once established, *H pylori* infection is diffusely present in the stomach, the degree of colonization is maximal in the antrum and inflammation varies accordingly (Stolte et al 1989). The progression of gastric atrophy once it has developed is more likely to be influenced by environmental and genetic factors (deLuca et al 1992).

2.3 NUD and *H pylori*

Non-ulcer dyspepsia is a persistent or recurrent pain or discomfort centred in the upper abdomen, where there is no evidence of a structural disease, such as peptic ulceration or malignancy, that is likely to explain the symptoms (Talley et al 1991). This condition is quite common and the majority of cases go undocumented.

To establish a causal relationship between *H pylori* and NUD, there should be convincing evidence that the infection is more common in dyspeptic patients, and precedes the development of symptoms, and that eradication of infection leads to loss of symptoms in the long term. The prevalence of *H pylori* infection in Europe and other Western nations is higher in older patients and among lower socioeconomic groups. *H pylori* is reported to be present in about 50% of patients with NUD but the role of this organism in the pathogenesis of NUD remains uncertain (Talley et al 1988, 1991 and 1993; 1993).

Evaluation of controlled trials is difficult because of the heterogeneous nature of NUD, and because of differences between studies in the assessment of *H pylori* infection, and the fact that patients with NUD are subject to a strong placebo response. Kemmer et al (1994) reported that patients with NUD have clinical features that appear to help distinguish between those with and without *H pylori* infection. In the study, the total dyspepsia symptom score was higher in *H pylori*-infected patients with NUD than in uninfected patients. This score was based on five factors-

pain relief after food intake, body weight, past history of an ulcer, and absence of diarrhoea and fat intolerance. In this way, patients with, for instance, irritable bowel syndrome, were carefully excluded (Talley NJ et al 1987). Limitations to this, not surprisingly, have occurred when similar scoring systems identified organic diseases in patients presenting for endoscopy (Talley et al 1993).

The difficulty in identifying characteristic symptoms in patients with NUD has been highlighted in other studies (Schubert et al 1992, Sobala et al 1990). The largest study to date is from the USA, where Schubert et al, found that, in 474 patients, neither the presence of the infection nor the activity of gastritis were related to specific symptoms, including pain, nausea and bloating. Moreover, the seroprevalence of *H pylori* has been found to be similar in patients with NUD who have symptoms suggestive of peptic ulceration, symptoms suggestive of gastroduodenal motility disorder, or non-specific complaints (Holtmann et al 1994). Studies which found that one or more specific symptoms were associated with the infection have been generally inconsistent (Bernersen et al 1992, Marshall et al 1984, Rokkas et al 1987).

If *H pylori* is a cause of NUD, therapy directed at eradicating the organism should lead to loss of symptoms in the long term. However, this is not the case. The trials of therapy for *H pylori* in NUD have produced, at best, mixed results (Lambert 1993, Talley 1993).
In some studies, *H pylori* was successfully eradicated in 50% of cases, though symptom scores measured four weeks after the end of treatment improved both in patients in whom eradication was successful and in those who remained infected. However, when measured one year after treatment, symptom scores remained low in patients in whom the organism had been eradicated, but returned to pre-treatment levels in

infected patients; a significant number of patients in whom the organism was not eradicated also went on to develop duodenal and gastric ulcers (Bernersen et al 1992).

Symptom relief following *H pylori* eradication in NUD can be explained by changes in gastric acid secretion which may suggest that NUD is probably a discrete disease which forms part of a spectrum of ulcer-related disease. At present, the role of *H pylori* in the aetiopathogenesis of NUD is not conclusive. What is certain, however, is that patients presenting with dyspepsia who are *H pylori*- positive have a much higher incidence of peptic ulcer and gastric cancer than those who are *H pylori*-negative.

2.4 Gastric neoplasm and *H pylori*

Gastric cancer is the fourth most common cause of death from cancer in the UK and accounts for about 10,000 deaths each year in the UK. The widespread use of endoscopy has facilitated its early diagnosis in some countries, with improved survival rate (Sue-Ling GM et al 1993). It has a higher prevalence in Japan, China, Colombia and Finland, though over the last fifty years, prevalence has fallen sharply in the developed world.

The association between bacterial infection and carcinoma has been known for a long time. In the 1850s, both Velpeau in France and Virchow in Germany maintained that cancers arose from local influences such as infection and inflammation. Known examples are chronic infection and bladder carcinoma, *Salmonella* infection and cholangiocarcinoma, *S. bovis* and colorectal cancer.

Several other risk factors have been identified including smoking, a high salt diet, low socio-economic class, blood group A and previous atrophic gastritis. An association between *H pylori* infection and an increased risk of gastric cancer was suggested after histopathological examination of gastric biopsy specimens showed that *H pylori* infection was more common in patients with gastric cancer than in those without pathological lesions.

A meta-analysis of three studies of the association between *H pylori* and gastric cancer concluded that there was an approximate 9-fold relative risk association between *H pylori* infection and gastric cancer (Foreman et al 1994). A case-control study which looked at serum antibodies from a number of index cancers showed not only was there a higher rate of seropositivity in cancers than controls (69% vs. 47%), but that antibody concentrations in the cancer patients were considerably higher (90 ug/mL vs. 3.6 ug/mL) (Forman et al 1991). This study estimated an odds ratio for risk of gastric cancer in those infected with *H. pylori* of about 2.8.

In the EUROGRAST study (1993) of associations between *H. pylori* and gastric cancer, a total of 17 populations in 13 different countries were examined. Serum antibody tests were performed in a single centre as the index of infection with *H. pylori*. Prevalence rates of *H pylori* seropositivity were related to local gastric cancer incidence. The conclusion was that a 100% infection rate with *H pylori* conferred about a six-fold risk of gastric cancer compared with a similar population with no infection (Eurograst Study Group 1993).

H pylori infection is thought to be associated with all types of gastric cancers with the exception of tumours of the gastric cardia.

Adenocarcinoma of the stomach has two histological variants: the intestinal, well differentiated form; and the diffuse poorly differentiated form which carries a worse prognosis. There are substantial variations world-wide, though. Some areas in China have contrasting high and low incidences of gastric cancers even though the overall prevalence of *H pylori* infection is high; while in Africa, there is a high prevalence of *H pylori* yet gastric cancer is rarely reported. Finding low rates of cancer in populations with high infection rates may be due to differing patterns of *H pylori* gastritis seen in individuals with varying levels of acid production. (Forman 1996).

It has also been shown that gastric mucosa-associated lymphoid tissue was a direct result of *H pylori* infection and perhaps provided the background to the development of gastric lymphoma (Wotherspoon et al 1991). The stomach is the most common extra-nodal site for non-Hodgkins lymphoma (NHL). NHL is almost invariably a B-cell tumour with histological characteristics closer to those of mucosa- associated lymphoid tissue (MALT) than those of lymph nodes. The stomach is normally devoid of lymphoid tissue and only acquires MALT in the presence of chronic *H. pylori* infection. Prevalence studies have shown that *H pylori* is greater in those areas of the world that have a high incidence of gastric NHL, and virtually all patients with NHL have evidence of *H pylori* infection. Retrospective examination of the gastric mucosal biopsy specimens from patients with evident gastric MALTomas demonstrated chronic active gastritis associated with *H pylori* infection in 98.4% of samples taken some distance from the tumour and the infection may represent a pre-lymphoma condition (Graham et al 1993).

There are several possible mechanisms by which *H. pylori* may be involved in gastric cancer. Infection of the stomach produces a destructive lesion of the mucinous surface of the epithelium, beginning as acute and chronic gastritis, progressing to chronic atrophic gastritis, intestinal metaplasia and dysplasia. When atrophic gastritis becomes severe and extensive, hypochlorhydria ensues. This favours the appearance of bacterial growth, nitrites and nitroso compounds in the gastric lumen. These compounds probably induce a premalignant gastric lesion (Recavarren-Arce et al 1991). Potentially mutagenic pathways include the roles of the bactericidal reactive oxygen metabolites (ROM) and N-nitroso compounds (NOC). Acute infection of *H. pylori* results in prolonged inhibition of naturally occurring ascorbic acid, thereby permitting the formation of N-nitroso compounds (NOC) (Sobala et al 1991). Oxidative stress from ROM damage has a proven link with carcinogenesis, operating via structural changes in DNA activation, activation of cytoplasmic and nuclear signal transduction pathways, and modulating the activities of stress genes and stress proteins. There is increased ROM production and lipid peroxidation in *H. pylori* - positive patients, levels of which fall to normal after successful eradication of the infection.

In 1994, the World Health Organisation (WHO) added *H pylori* to its list of known carcinogens. Proving the causal relationship has been more difficult for cancer than for ulcers. While there is evidence for the association between *H pylori* and gastric tumours on one hand, and between *H pylori* and ulcers on the other, the negative association between DU and gastric cancers makes it less conclusive. There are several populations such as in China (Forman et al 1990) and Africa (Megraud et al 1989) where *H pylori* infection is prevalent at young ages but gastric cancer is uncommon. Also, *H pylori* does not appear to

influence the site of gastric cancer or its histological type (diffuse or intestinal), showing lack of specificity (Sipponen et al 1992).

2.5 Oesophagitis and *H pylori*

While there is convincing evidence from research studies associating *H pylori* infection with ulcer and non-ulcer dyspepsia, the relationship between *H pylori* and oesophagitis is scanty. As in other parts of the gastrointestinal tract, *H pylori* has been found in the oesophagus. Normal oesophageal mucosa, unlike the gastric antral mucosa, is not a site of predilection of *H pylori* (Walker et al 1989) and studies have shown no significant difference between patients with and without oesophagitis (Agnholt et al 1991).

However, in Barrett's oesophagus the prevalence of *H pylori* was higher (23.8%) than in patients without Barrett's (nil) (Kogan et al 1992). The implication of this is that *H pylori* infects gastric type mucosa only and cannot be of aetiological importance in oesophagitis.

2.6 Eradication therapy and re-infection

Half the world's population is believed to be infected with *H. pylori.* The importance of accurate selection of the appropriate patients for eradication therapy is, therefore, vital as we cannot expect to treat every infected person, especially in the absence of any associated pathology. The 1994 United States National Institutes of Health Concensus Conference recommendations for *H. pylori* eradication encompass patients with duodenal or gastric ulcers unrelated to the use of non-steroidal anti-inflammatory drugs (NIH Consensus conference 1994). These recommendations assumed that ulcers not caused by NSAID were *H.pylori*-related. It provided an important watershed in the acceptance by the medical community that *H.pylori* infection is an important clinical problem. The inadequacy was the fact that a large group of symptomatic *H. pylori*-infected subjects, unrelated to ulcers, was not addressed. Thus treatment was restricted to patients with a past or present history of peptic ulceration. It supported neither treatment of infected patients with non-ulcer dyspepsia (Veldhuyzen van Zanten 1997), nor eradication as a prophylactic measure against diseases such as gastric cancer (Uemura et al 1996), gastric lymphoma, peripheral vascular disease, or even stroke with which *Helicobacter pylori* has been linked.

These guidelines were refined at the 1996 Maastricht conference (Malfertheiner et al 1997). In addition to the NIH recommendations, the updated guidelines of the Maastricht conference added that in *H. pylori*-positive ulcer disease complicated by acute haemorrhage, eradication should start at the same time as oral feeding. It also strongly recommended treatment in patients with low-grade mucosa-associated lymphoid tissue (MALT) gastric lymphoma, gastritis with severe

macro- or microscopic changes and after resection of early gastric cancer.

There is still lack of hard scientific evidence of the benefits of treatment in some group of patients: patients with non-ulcer dyspepsia; atrophic gastritis; long-term treatment with proton-pump inhibitors for gastro-oesophageal reflux disease or NSAID treatment. In the group of patients with non-ulcer dyspepsia, therapeutic trials have failed to show a statistically significant acute clinical benefit (Veldhuyzen van Zanten 1997). In a further review of 16 trials, 8 suggested a benefit from treatment, while 8 did not (Tolley 1994). The general lack of consistency of these trials probably reflects selection and the true definition of 'functional dyspepsia'. Moreover, peptic ulceration is a disease characterized by relapse and remission, and a number of non-ulcer dyspeptics must be so diagnosed because their endoscopy was performed during remission (Pounder et al 1997).
Helicobacter pylori infection may also result in chronic active gastritis. Successful eradication of *H pylori* in this condition has been shown to result in histological resolution. Although treatment has been advised in this condition, the benefit in the long term is not known. It is believed that majority of patients with *H. pylori* gastritis undergo spontaneous remission and only a few progress to peptic ulcer and other *H. pylori*-induced conditions.

NSAID use has not been proven to interact with *H. pylori* to increase the risk of peptic ulceration. Most available studies reviewed suffered from a suboptimal design and small sample sizes. The general consensus is that an interaction between infection and NSAID use is unlikely to be of any clinical relevance (Kuipers et al 1995).

Eradication therapy is also advised in gastric cancer and lymphoma prevention or after gastric surgery for carcinoma. A group of patients who were found to have an early intestinal type of gastric cancer were studied. Those patients who received eradication treatment following endoscopic resection of these lesions had remission of intestinal metaplasia, and no new cancers developed during a mean 24 month follow-up. Of the patients who did not receive treatment, 9% developed a new gastric cancer during similar follow-up (Uemura et al 1996). Despite these studies, there has not been decisive proof that treatment decrease the chance of later development of gastric malignancy. Such proof can only be obtained after a long term follow-up and as part of a planned treatment programme (Isaacson 1994).

Proton pump inhibitors have been implicated in causing an increase in the activity of gastritis in the body of the stomach of subjects infected with *H. pylori* (Lamberts et al 1993). Even a short course of treatment with omeprazole appears to produce long-lasting changes (Hackelsberger et al 1996). It has been recommended that patients with reflux oesophagitis and *H. pylori* infection should receive treatment to eradicate *H pylori* before embarking on long-term treatment with a proton pump inhibitor. However, this recommendation has been criticized at a recent meeting of the US Food and Drug Administration Gastroenterology Advisory Panel for lack of adequate evidence.

CHAPTER THREE
ENDOSCOPY- INFECTION AND DISINFECTION.

3.1. Endoscopy- infection and disinfection

In the last few decades, the important developments that have occurred in the study of peptic ulcer disease can largely be attributed to the development of the flexible fibreoptic endoscope and its increased diagnostic and therapeutic purposes. It has also been recognised that the use of contaminated endoscopes can lead to transmission of infection. The endoscope can be colonised in storage by opportunistic infections such as *Pseudomonas*, and this may be inoculated into a succession of patients. However, it is presently not clear whether infection can be transmitted from one site to another in the same patient at the time of endoscopy, with the endoscope acting as the vehicle of transmission.

Disinfection of fibreoptic endoscopes can be inadequate. An outbreak of infection by *Salmonella* species involving a total of 81 patients was attributed to contaminated endoscopic equipment which had not been effectively disinfected. No new cases of Salmonellosis were reported and endoscope cultures became negative when the disinfectant was changed to povidone-iodine or 2% glutaraldehyde (O'Connor et al).

Apart from *Salmonella* species, other microorganisms such as *Strongyloides, Mycobacterium tuberculosis Hepatitis B virus* *Pseudomonas* have been reported.
It is clear that the possibility of transfer of microorganisms by the endoscope exists if adequate preventive measures are not taken. Endoscopic equipment invariably becomes contaminated with blood, saliva, and bile during routine use. These are probably the main vehicles of transmission of infection.

Contamination of endoscopy room and personnel with enteric flora occurs easily during endoscopic procedures. Any such risk of cross-infection should be eliminated by fastidious disinfection of the endoscopic equipment.

CHAPTER FOUR

PLAN OF STUDY

4. PLAN OF STUDY

Approval to carry out this study was obtained from the joint University College London / University College Hospitals Committee on the Ethics of Human Research.

The primary aim of this thesis was to study the aetiology of DU in relation to infection of the stomach with *Helicobacter pylori.* Is *H pylori* infection the principal aetiological factor or simply an associated factor in peptic ulcer disease? Moreover, does the infection precede the ulcer?

One line of investigation (A1) was to identify the various factors associated with *Helicobacter pylori* and those associated with DU, independently. This was done by a retrospective analysis of the records of patients undergoing endoscopy in the routine endoscopy clinics of The Middlesex and University College Hospitals during the periods 1992-1995. It is emphasised that these patients had been treated previously and were never seen by me. The statistical technique of logistic regression analysis was applied to the data collected.
A preliminary analysis of this study yielded more questions than answers with respect to *Helicobacter pylori* as the principal aetiological factor in DU. It indicated, amongst other conclusions as enumerated later, that there might be a link between the presence of *H pylori* and the number of times a patient had undergone endoscopy.

Arising from this study, an investigation was later performed to test the relationship between endoscopy and *Helicobacter pylori* infection. Also, following this conclusion, a study of the efficacy of sterilisation of endoscopy equipment was performed (A2).

The next investigation was designed to investigate whether passage of the microorganism from the mouth to the stomach could be demonstrated. Several studies have demonstrated that the usual site of *Helicobacter pylori* colonization in humans is the pyloric antrum. To get to this site of predilection, it is hypothesised that the organism must pass via the mouth. This investigation B also enabled me to study the prevalence of *Helicobacter pylori* in a group of patients presenting with 'dyspepsia'.

Lastly (Investigation C), I followed up some of these patients by breath test. In this way, I was able to determine if endoscopy or any other factor had influenced *H. pylori* status after 6-12 weeks.

In order to facilitate interpretation, the method and the result of each of the steps is described in chapter five.

CHAPTER FIVE

METHODS AND RESULTS

5. METHODS AND RESULTS

5.1 Investigation A1

Logistic regression analysis of factors associated with *H pylori* and DU

Method:

Records of patients who underwent upper gastrointestinal endoscopy in the endoscopy clinics of the Middlesex and University College Hospitals in 1993/4 were studied retrospectively in order to elucidate factors associated with *Helicobacter pylori* infection, and with DU.
450 case-notes of unselected patients, i.e. all the patients who had attended except for those whose records were unavailable, were retrieved and information obtained for each performance of endoscopy as follows;

1) age, sex, smoking status, hospital number, serial number of endoscopy

2) endoscopic diagnosis made by the endoscopist on that occasion, recorded as duodenal ulcer (DU), oesophagitis (Oeso), non-ulcer dyspepsia (NUD), other macroscopic lesions (Other)

3) *Helicobacter pylori* status recorded as:
(a) positive (+), negative (-) or unknown,
(b) at the time of endoscopy (hp),
(c) immediately before endoscopy (hpmm),

(d) at any time previous to the endoscopy other than immediately before (prehp).

4) anti- *H pylori* treatment whether-

(a) received treatment (+), did not receive treatment (-) or unknown,

(b) immediately before the endoscopy (treatment)

(c) at any other time before the endoscopy (pretreat)

Data were collected on spreadsheets and analysed with logistic regression analysis using SPSS.

Results:

There were 661 episodes of endoscopy involving 450 patients. In 302 of the episodes the patient was female, in 359 male. In 253 of the episodes the patient was a smoker, in 312 a non-smoker, and in the remaining 96 cases smoking status was not recorded.

H pylori status was negative in 193 episodes, positive in 209, and unknown in 259.

Smoking remained strongly associated with DU. Oesophagitis was negatively associated with *H pylori*. It is interesting however, that the number of endoscopies a patient had undergone positively influenced *H pylori* status.

The nature of this link between *H pylori* infection and the number of endoscopies is uncertain.

(See tables 4 and 5).

5.2 Investigation A2
Test of adequacy of endoscopy sterilization

This investigation was performed to test the efficacy of sterilisation procedures for endoscopes. The possibility of endoscopic transmission of microorganisms has been discussed in Chapter 3. The aim of this test was to examine whether our endoscopic cleaning procedure for *H. pylori* adequately sterilised the instruments.

At present several tests are available for the detection of *H. pylori* in clinical samples. Several investigators found PCR to be more sensitive than culture or the other routine laboratory tests.

Method:

Twenty (20) consecutive patients from the routine endoscopic clinic, who tested positive to the Rapid Urease Test (RUT) and three (3) who tested negative to the RUT, were included in this study. The three patients acted as the control group. Endoscopy was performed with Olympus XQ20S. Gastric antral biopsy specimens were taken with standard biopsy forceps for RUT.

After each endoscopic procedure, the working channels of the endoscope were flushed with 10 ml distilled water and the effluent collected in a sterile universal container, labelled 'pre-sterilisation (nos.1-20)' .
The endoscope was then cleaned and disinfected by means of combined manual and machine cleaning and disinfection. For this part of the

experiment, the Nursing Staff responsible for the cleaning procedure were not aware of the test of endoscopy sterilisation being carried out. After the sterilisation, the working channel of the endoscope was again flushed with 10 ml distilled water and the effluent collected as 'post-sterilisation (nos. 1-20)'.
The specimens were transferred to the molecular biology laboratory for DNA extraction and polymerase chain reaction (PCR) as described later.

Results:

Control group
Of the three patients who were *H pylori*-negative by the RUT, all the pre- and post-sterilisation specimens remained *H pylori*-negative to the PCR.

Study group
Of the 20 patients who were *H pylori*-positive by the RUT, 12 were *H pylori*-positive pre-sterilisation; while none was *H pylori*-positive post-sterilisation as tested using PCR. See table 6.

5.3 Investigation B

Detection of *H pylori* from oral cavity and gastrointestinal tract of dyspeptic subjects.

5.3.1 Test design:

Patients attending with symptoms of dyspepsia were investigated at endoscopy, looking for *H. pylori* in the mouth as well as in the stomach and duodenum. A questionnaire was given before the endoscopy to determine the patient's age, gender, ethnicity, hospital number, smoking status, length of history, previous endoscopy and diagnosis, past medical history, and drug history including eradication therapy. The symptomatology and endoscopic findings were also recorded.

5.3.2 Patient selection:

Patients referred for routine upper gastrointestinal endoscopy for investigation of their symptoms of dyspepsia were recruited into the study. In accordance with the guidelines of the local Committee on Ethics and Human Investigation, an information sheet explaining the procedure involved and a form for informed consent were given to patients before endoscopy.

A total of 208 unselected patients participated in the study. Exclusion criteria were: voluntary; patients with potential infection risks such as hepatitis; bleeding tendency; antibiotic or other anti- *H pylori* therapy in the preceding 8 weeks or those who required antibiotic prophylaxis.

5.3.3 Subject preparation:

Patients were requested to have nothing to eat or drink for at least 8 hours before the procedure. On admission to the Endoscopy suite, a clinical questionnaire was completed, giving information about patient's personal details as enumerated above. The test was explained to the patient and where sedation was used for the procedure, it was arranged that someone would accompany the patient home.

5.3.4 Equipment and performance of upper gastrointestinal endoscopy:

All equipment used in The Middlesex Hospital Endoscopy Suite where this examination took place, satisfied the Health and Safety standards and were regularly checked and updated. This is a large referral unit where diagnostic and therapeutic endoscopies are performed routinely every week day.

The patient was positioned semi-prone, false dentures were removed and a mouth-guard was securely placed between the lips. The patient was allowed to choose an intravenous midazolam sedation and/or throat spray with xylocaine. Once the patient was comfortable, the examination was carried out with the passage of the endoscope, Olympus XQ 20S. Diagnosis was established and specimens collected for further tests.

5.3.5 Recovery and after-care of patients:

In the recovery room, patients were monitored until the effect of the sedation had worn off. In deeply-sedated patients, the effects were reversed with intravenous annexate and they were discharged home in a satisfactory clinical state in the company of a relative or friend. Those who received the xylocaine throat spray were not allowed to feed until throat sensation had returned (usually 30 minutes). An advice sheet and an appointment to return for ^{13}C- urea breath test were given for a later test in 6-12 weeks (described later).

5.3.6 Specimen collection:

5.3.6.1 Collection of oral specimens

Oral specimens were always collected prior to the endoscopic procedure in order to exclude the possibility of contamination from the gastrointestinal tract during the withdrawal of the endoscope.

5.3.6.1a *Saliva*

Saliva specimens were collected from 208 patients before the endoscopy. The patient was asked to spit 2-3mls. of saliva directly into a new sterile 5 ml blue-top eppendorff container. This was transferred to the laboratory and stored at -70 ^{0}C until later tested.

5.3.6.1b *Dental plaque*

This aspect was supervised by a Dentist who was involved in the study. Using a sterile periodontal curette, both supra- and sub-gingival plaques were collected by a gentle upward scrape against the tooth surface. The material was immediately placed in a sterile tube containing normal saline and then stored at -70 ^{0}C.

5.3.6.1c *Tongue and oropharyngeal swab*

The rest of the oral cavity, particularly, the tongue fissures and the oro-pharynx, were stroked with a sterile microbiology swab stick which was then immediately replaced in the culture medium.

5.3.6.2 Collection of gastrointestinal specimens:

5.3.6.2a *Gastric juice*

To prevent contamination, gastric juice was collected before any biopsy was taken. A fibre-optic endoscope was passed down through the mouth and a 10 mL volume of gastric juice was aspirated with the endoscope and collected via a trap collector tube into a sterile container. This was buffered to a neutral pH with an equal volume of Tris and frozen at -70 ^{0}C.

5.3.6.2b *Gastric antral biopsy*

Three antral mucosa biopsy specimens were taken with forceps. In cases with the endoscopic appearance of definite pathology, a further biopsy was also taken for histological confirmation.

5.3.6.2c *Duodenal aspirate*

Secretion in the second part of the duodenum was aspirated in 60 patients in whom an adequately large volume could be collected. The first aliquot was discarded to exclude possible contamination. 10-20 mL were subsequently collected and tested.

5.3.7 Transport and storage:

All specimens were properly labelled for easy identification and transferred to the laboratory. Specimens for culture were processed immediately, while the remainder were stored at -70 ^{0}C for subsequent analysis.

5.3.8 Diagnostic tests performed:

5.3.8.1 Rapid Urease Test (RUT)

For preliminary diagnosis of *H. pylori*, one antral biopsy specimen was obtained at endoscopy and immediately placed in urea broth for RUT.

5.3.8.2 Culture

Each of the specimens from the dental plaque, saliva, oropharyngeal swabs, gastric juice, antral biopsy and duodenal aspirate were inoculated onto plates containing Columbia Base agar with 5% horse blood and 2 μg/ml amphotericin B. Each plate incubated at 37 ^{0}C under microaerophilic conditions until colonies were seen. The culture was considered negative if a colony did not develop within seven days. As soon as a colony grew, identification of *H pylori* was carried out by Gram coloration and by biochemical tests. When colonies were Gram negative and showed positivity on catalase, urease and oxidase tests, curved microorganisms were identified as *H pylori.*
(This test was carried out by Dr. Holton, Consultant Microbiologist).

5.3.8.3 Histology

An antral biopsy specimen and a biopsy from the endoscopic lesion where appropriate were fixed and stained with haematoxylin-eosin and giemsa. The presence on the mucosal surface of curved rod-shaped micro-organisms resembling *H pylori* that stained with haematoxylin-eosin or giemsa was considered positive.

5.3.8.4 Polymerase chain reaction (PCR)

PCR was carried out in the microbiology laboratory in an area designated for this purpose only. Because of the extreme sensitivity of this procedure, a strict sterile protocol was adhered to in order to avoid false amplification and false results.
The overall experimental protocol involves the following steps:-

1. CLINICAL ISOLATES- dental plaque, saliva, tongue and oro-pharyngeal swabs, gastric juice, gastric biopsy, duodenal aspirate
2. DNA EXTRACTION
3. PCR MIX AND REACTION CYCLE
4. GEL ELECTROPHORESIS AND PHOTOGRAPHY
5. RESTRICTION ENDONUCLEASE

5.3.8.4a Clinical isolates

All the specimens collected, viz dental plaque, saliva, oropharyngeal swab, gastric juice, gastric antral biopsy and duodenal aspirate were analysed with PCR.

5.3.8.4b DNA extraction

The isolation of genomic DNA from the clinical isolates was done in an area in the laboratory designated for this purpose, away from the PCR mix and reaction area. The method I used was based on the instructions

in the Puregene DNA isolation kits supplied by Gentra Systems, Inc., (catalog number D-T200).

General principle

Purified genomic DNA is required in DNA analysis procedures involving PCR. This method relies on nucleated cells as a source of genomic DNA. Nucleated cells are found in whole blood, bone marrow, body fluids, animal and plant tissues, cultured cells, bacteria etc. The cells are first lysed in the presence of a DNA preservative which solubilises the cellular components. This preservative works by limiting the activity of enzymes that are contained in the cell and the cellular environment and are capable of digesting DNA. Contaminating RNA is removed by treatment with an RNA- digesting enzyme. Contaminating cytoplasmic and nuclear proteins are removed by salt precipitation. The pure genomic DNA is finally isolated by precipitation with alcohol and dissolved in a buffered solution containing a DNA preservative.
Materials required- Cell lysis solution; protein precipitation solution; DNA hydration solution; Rnase A solution are all supplied in the extraction kit. Others are isopropanol; 70% ethanol; micropipettors and pipettes; sterile microfuge tubes and microfuges; vortex mixer; water bath. The Rnase A solution is stored at 4 ^{0}C while other reagents are stored at room temperature.

I followed the procedure of DNA extraction as recommended by the manufacturers:

a) Sample processing

Clinical specimens frozen at -70 ^{0}C were thawed and prepared for DNA extraction and subsequent amplification. Freezing and thawing the specimen leads to liberation of sufficient DNA for amplification. Insoluble material from the dental plaque and the gastric biopsy were

harvested by centrifugation at room temperature for 10 minutes in a microcentrifuge. The supernatant was decanted and the precipitate was homogenized using 30-50 strokes with a microfuge tube pestle. 1 ml of the soluble homogenate was placed in a 1.5 ml tube and centrifuged at 16,000 x g for 5 seconds to pellet cells. The tongue and oropharyngeal swabs were dipped into distilled water to wash off the isolate. This was then centrifuged at 16,000 x g for 60 sec. and a soluble homogenate obtained. The other clinical isolates (saliva, gastric juice and duodenal aspirate) were soluble materials which were processed directly.

b) Cell lysis

600 µl of cell lysis solution was added to the pellet cells and was gently pipetted up and down until all the cells were suspended. This was then incubated in the water bath at 80 ^{0}C for 5 minutes to lyse the cells. The solution was cooled to room temperature.

c) RNase treatment

3 µl of RNase A solution was added to the cell lysate in order to ensure that any contaminating RNA molecules were completely digested. The mixture was vortexed vigorously and incubated at 37 ^{0}C for 60 minutes.

d) Protein precipitation

The Rnase A- treated solution was left to cool to room temperature. 200 µl of protein precipitation solution was added and vortexed vigorously at high speed for 20 seconds to obtain a uniform mixture. This was then centrifuged at 16,000 x g for 3 min. to pellet the precipitated protein. The supernatant contains the DNA.

e) DNA precipitation

The supernatant was gently poured into a clean 1.5 ml tube containing 600 µl 100% isopropanol. The mixture was centrifuged at 16,000 x g for 60 seconds, separating the DNA as a small white pellet. The supernatant was discarded and the tube drained onto a sheet of clean absorbent paper.

600 µl 70% ethanol was added to wash the DNA pellets which were then centrifuged at 16,000 x g for 60 sec. The ethanol was then gently poured off. The tube was drained onto clean absorbent paper and allowed to dry in the air.

f) DNA hydration

The DNA was allowed to become rehydrated by adding 100 µl DNA hydration solution and left overnight at room temperature.

The yield of DNA obtained depended on the number of cells in the sample. It is estimated that 100 µl will give a concentration of 500 µl/ml if the yield is 50 µg.

5.3.8.4c PCR MIX AND REACTION

The components of the PCR mix included the following:

(1) Primers

The primers used consisted of two 20- base, HP1 and HP2, synthesised commercially (Oswel DNA Service, UK. Sequence numbers V1777 and

V1778 respectively.). The primers for the specific detection of *H pylori* were designed for areas of the 16S rRNA gene in which there is the least sequence homology.
HP1 and HP2 together amplified a 109-base pair PCR product.
The primers were supplied in 1 ml solution as;

HP1 - 5' CTG GAG AGA CTA AGC CCT CC 3'
HP2 - 5' ATT ACT GAC GCT GAT TGT GC 3'

(2) Nucleotides
These were purchased from Perkin Elmer Corp. and stored at -70 ^{0}C. Individual nucleotides are not stable when stored diluted because the phosphate portion dissociates. A mix of all four nucleotides at a concentration x10 was therefore prepared to avoid thawing and re-freezing the stock solutions more than necessary. For optimal results, 200 µM (concentration) of each of the nucleotide was used which worked out in the final master mix volume as 16 µl in a 1:10 dilution.

(3) PCR buffer
The buffer was used as 10 µl of 10X PCR buffer per tube which contained a final concentration of 10 mM Tris-HCl, pH 8.3, 50mM KCl.

5.3.8.4d MASTER MIX

A master mix of reagents for all samples was prepared first and then aliquots were apportioned to individual tubes. Amplifications were performed in 100 µl of reaction mix in capped polypropylene reaction tubes. These tubes provide the best heat transfer and uniformly fit in the wells. Since DNA may stick to plastics and since nucleases are often found on surfaces, thus leading to false amplifications, all the tubes and pipette tips used in the PCR reaction were sterile. The component of the master mix was as follows:-

1. Ultrafiltered water
2. 10x PCR buffer
3. Nucleotides- dATP, dCTP, dGTP, dTTP
4. Primers HP1 and HP2.
5. Amperase UNG
6. Amplitaq DNA polymerase
7. $MgCl_2$
8. Experimental templates i.e. extracted DNA.

OPTIMIZATION OF THE PCR COMPONENTS

Before the performance of PCR on the extracted DNA, the volume and concentration of each of the components of the PCR were determined empirically based on a series of initial experiments. This step is necessary in order to find the optimum concentration that gives the best product yield.

The principles employed were as follows:

1. The total volume of the reaction always equals 100 µl per PCR tube and the volume of the ultrafiltered water can be varied as long as the final reaction volume remained 100 µl.

2. A series of dilution of a known *H. pylori* genomic DNA was used to determine the sensitivity of the reaction. 1 ng of the template was diluted as $x10^{0}$; $x10^{-1}$; $x10^{-4}$; $x10^{-8}$. Each dilution was amplified.

3. The optimal magnesium chloride concentration was varied in the range of 1.0 - 4.0 mM in the reaction mix. A concentration of 2.0 mM gave the best yield in terms of the intensity of the bands obtained after electrophoresis on agarose gel.

4. The primer concentrations were varied between 1.0 - 2.0 mM each.

5. AmpliTaq and AmpErase were used as 1.0 µl each and were not varied throughout the experiments.

6. A positive control from known *H. pylori* DNA was employed throughout the experiment. Negative controls were used from an *E. coli* strain as well as by adding distilled water instead of a template DNA.

Experiment 1- Determination of the optimal $MgCl_2$ concentration

Reaction mix:	Tube1 (µl)	Tube2 (µl)	Tube3 (µl)	Tube4 (µl)
x10 PCR buffer	10	10	10	10
Nucleotides(at 200mM)	16	16	16	16
AmpErase	1	1	1	1
Primer HP1	10	10	10	10
Primer HP2	10	10	10	10
AmpliTaq	1	1	1	1
$MgCl_2$*	4	8	12	16
DNA template	5	5	5	5
Water	43	39	35	31
Sterile mineral oil	50	50	50	50
Total volume	100	100	100	100

*Four different concentrations .

Experiment 2 - Determination of the sensitivity of the reaction.

In this experiment, the volume of the other components of the PCR mix was constant. The DNA template was taken from a known colony of *H. pylori* strain. This colony was diluted with distilled water. 5µl of each of the diluted volume was then used as the template in the PCR reaction.

Reaction mix	µl
X10 PCR buffer	10
Nucleotides	16
AmpErase	1
Primer HP1	10
Primer HP2	10
AmpliTaq	1
$MgCl_2$	8
DNA template*	5
Water	39
Sterile mineral oil	50

*DNA template diluted at $x10^{0}$, $x10^{-1}$, $x10^{-4}$, $x10^{-8}$

PCR REACTION CYCLE

A two-step PCR was used for the cycling programme. The conditions were varied and an optimal yield which best reconciled economical running costs with maximum specificity and sensitivity was chosen. PCR tubes were placed in the automated machine (Hybaid cycler, Omnigene), and the reaction cycle programmed.

First stage:

Step 1. Melting step - (to separate the complementary strands of DNA) set at 95 ^{0}C for 30 sec

Step 2. Primer annealing step - (to allow hybridization of the primers to the ssDNA and initiation of polymerisation) set at 60 ^{0}C for 30 sec

Step 3. Primer extension step - (to complete the copy initiated during annealing) set at 72 ^{0}C for 30 sec
Steps 1,2,3 were run for 39 cycles.

Second stage:

95 ^{0}C for 30sec
60 ^{0}C for 30sec
72 ^{0}C for 5min
The second stage was run for 1 cycle

Post amplification handling:
After the final cycle, samples were held at 72 ^{0}C until they were removed from the instrument system. The tubes were then quickly transferred into frozen ice at -20 ^{0}C in order to denature any remaining AmpErase UNG activity.

5.3.8.4e AGAROSE GEL ELECTROPHORESIS OF PCR PRODUCTS

Preparation of samples

The sterile PCR products were kept on ice while new tubes were placed in a rack for the addition of the loading buffer and the PCR molecular markers. These reagents were purchased from Sigma Biosciences (catalog numbers P- 7206 and P- 9577 respectively).

The PCR marker consisted of fragment sizes 50 - 2000 base pairs and was supplied in x1 PCR loading buffer. I followed the recommended usage as suggested by the manufacturers.

1 µl of the PCR loading buffer was added to each tube including tubes for the positive and negative controls and were all labeled clearly. 5 µl of PCR product was added to each tube.

5 µl of PCR molecular marker was prepared for every batch of agarose gel run.

Preparation of x10 TBE buffer and agarose gel

x10 TBE was prepared using the following reagents:

Tris base	53.5 g
Boric acid	27.5 g
$Na_2EDTA._2H2O$	3.73 g
Ultrapure water	500 mls.

This was mixed well on the magnetic stirrer and subsequently used for preparation of the gel.

The agarose gel was prepared following these steps-

1 Add 45 ml ultrapure water to a small conical flask and chill on ice.

2 Weigh out 0.5 g agarose and add slowly to chilled water on magnetic stirrer.

3 Cover the conical flask with clingfilm and make a small hole in the centre to allow evaporation when boiling. Mark level of fluid on side of flask.
4 Dispense 5ml x10 TBE buffer and warm in 55 ^{0}C waterbath. Also warm some ultrapure water.
5 Place masking tape around gel tray to seal and place on flat adjustable glass plate with spirit level, in order to ensure a perfectly flat electrophoresis gel.
6 Choose combs with the correct number of slots for the samples.
7 Melt agarose by heating in the microwave oven in short bursts and avoid boiling over. If fluid drops below marked level, top up using warm ultrapure water with pasteur pipette.
8 Ensure agarose has dissolved completely and solution is clear. Allow to cool to 60 ^{0}C and add 5 ml x10 TBE. Use rubber holder to handle the hot flask.
9 Add 5 µl ethidium bromide solution, mix well avoiding creating air bubbles and pour onto sealed gel tray. Add combs and allow to cool for at least 15 minutes before proceeding to the electrophoresis.

Electrophoresis

x1 TBE buffer is prepared as described previously. The tape around the gel tray is removed and the gel tray is placed in a small electrophoresis tank.
15 µl ethidium bromide solution is added to 350 ml x1 TBE buffer and then gently poured into electrophoresis tank and over the gel.
Using fine non-sterile tips, PCR and control samples are carefully added to the wells. Tips used for PCR products are discarded to avoid cross-contamination.

The electrophoresis circuit is set to run from negative to positive terminal at 88 volts (8.8 volts/cm) for 30 minutes.

Photography

The electrophoresed gel and holder are removed to the dark room for the photography. The gel is photographed on the surface of ultraviolet light source, observing strictly the safety precautions.

5.3.8.4f RESTRICTION ENDONUCLEASE

Similarities between strains of *H. pylori* from the different sites in the same individual were determined using restriction endonuclease digestion with HaeIII. PCR products from 15 patients who tested positive for *H. pylori* in the mouth (results explained later) and corresponding GI sites, were digested.

A separate room different from where PCR was set up was used. HaeIII was purchased from SIGMA (Sigma Diagnostics, Poole, Dorset, U.K) The recommended working concentration and optimal incubation temperature of the restriction enzymes were determined.

Using sterile tips, PCR products from the 15 patients in whom *H. pylori* had been detected in dental plaque were dispensed into labeled tubes on ice.

The mix of water, buffer (provided with enzyme) and enzyme were vortexed and dispensed into these tubes. 20 µl mineral oil was added to each tube to prevent evaporation.

The tubes were placed in the water bath and incubated for 4 hours. After the incubation, the tubes were placed back on ice until it was time to run a gel.

Gel electrophoresis for digested PCR products required a harder gel. Agarose was prepared as previously described but using a concentration of 2%.
Electrophoresis was carried out at 8.8 volts/cm for 1 hour.

RESULTS

Two hundred and eight dyspeptic patients successfully took part in the study. (See tables 7 and 8)

DETECTION OF *H PYLORI* IN ORAL SITES

Saliva

Two tests were performed on salivary specimens, culture and PCR.
180 specimens were cultured - there was no growth of *H. pylori* in any of the culture plates after 7 days.
208 specimens tested by the PCR similarly did not detect any *H. pylori* DNA.

Oropharyngeal and tongue swabs

Swabs were cultured for 7 days and none of the 180 specimens yielded *H. pylori*. Also, PCR did not detect any *H. pylori* DNA in 208 swab specimens.

Dental plaque

2 of 180 specimens grew *H. pylori* after 5 days; while 15/208 or 7% were *H. pylori*-positive on PCR.

The 2 patients who were culture-positive had the characteristics as shown in tables 8 and 9.

15 dental plaque specimens (including the above 2) were positive on PCR. The endoscopic diagnosis in these patients was DU(8), GU(1), NUD(5) and gastritis(1). The overall breakdown of the affected patients is shown table 10.

Summary of results.

Culture and PCR of specimens of saliva, dental plaque, tongue and oropharyngeal swabs were obtained in 180 and 208 patients respectively. No salivary or swab sample was positive for *H. pylori* by any test. Two of 180 specimens of dental plaque were *H. pylori* positive by culture, and those two samples plus 13 more were positive by PCR. In the oral samples, there was never any evidence of *H. pylori* except in plaque. In 15 plaque samples (out of 208), PCR was positive for the organism, but in only 2 of these was the PCR result accompanied by a positive culture.

DETECTION OF *H PYLORI* IN GASTRIC AND DUODENAL SITES

Gastric juice

By culture, none of the 180 gastric juice plates grew *H. pylori* after 7 days. However, by PCR technique, 36 samples or 17% were *H. pylori*-positive.

Duodenal aspirate

This could only be collected in 50 patients because of technical difficulty in obtaining samples from an area distal to the first part of the duodenum.

None of the specimens was positive on culture.

6 or 12% were positive when tested with PCR.

Of the 6 patients, 4 had an endoscopic diagnosis of DU and one each of NUD and gastritis. All 6 had corresponding positive results from the gastric biopsy using PCR and all but one *H. pylori*-positive in the dental plaque.

Follow-up test of *H. pylori* status after 6 weeks by ^{13}C-UBT was available in 5 of these 6 patients, and were all positive.

Gastric antral biopsy

Multiple tests were applied to gastric biopsies; RUT, PCR, culture and histology. The latter was mainly to confirm endoscopic diagnosis where there was any lesion.

Many of these results overlapped considerably. This enabled me to compare the results of the various diagnostic tests

Using RUT criterion

208 specimens were tested for *H. pylori* by observing a change in the indicator colour of the urea broth.

At 1 hour or less, 69/208 were positive. A further 6 specimens were positive over the next 24 hours, giving a total of 75/208 or 36%. Results after 24 hours were not documented.

Using culture criterion

The specimens were incubated for 7 days and were tested for characteristic *H. pylori* growth in the medium. 61/180 or 34% specimens were *H. pylori*-positive. The remaining 28 specimens were erroneously left un-cultured.

Using PCR criterion

Amplified *H. pylori* DNA was run through agarose gel electrophoresis and observed under ultra-violet light. Results were obtained from specimens in all the patients (208). 114 or 55% were positive and 94 or 45% were negative.

Comparison of the diagnostic tests (see tables 11 and 12).

Culture and histology- There was 100% concordance between these two results when both were available.

Culture versus RUT- when culture was positive(61), RUT was positive in 54/61; and negative in 7/61. On the contrary, when culture was negative(119), RUT was negative in 115/119; and positive in 4/119.

Culture versus PCR

H. pylori was detected in all the positive culture results, and in some of the negative culture results.

61/180 antral biopsy-positive specimens were also positive by the PCR technique. 31 more were positive by the PCR, but negative by culture.

88/180 negative by culture were similarly negative by the PCR.

Prevalence of *H. pylori* in dyspeptic patients:

One of the aims of this study was to determine the relative frequency, in the different diagnostic categories, of *H. pylori* in patients undergoing endoscopy for the complaint of dyspepsia and to compare this with the control group .

I recruited 208 patients who had been referred for routine endoscopy for dyspepsia. Before the endoscopy, a questionnaire was filled detailing the following information; age, gender, ethnic origin, smoking habit, symptomatology, present medication.

At endoscopy, diagnoses were made and recorded. Tests were also carried out to determine *H. pylori* status based on PCR, RUT, culture and histology of endoscopic biopsies. The prevalence of *H. pylori* in the various endoscopic categories was determined in the 208 dyspeptic patients studied using RUT + culture results (see table 16).

The possible effect of a variety of factors on *H. pylori* prevalence was explored: age, sex, smoking, drugs, length of history, ethnic group. This information was obtained directly from the questionnaire filled in by the patients before endoscopy.

Frequency of *H. pylori* in different age groups (see tables 19 and 20):

This result showed an increasing prevalence with age and low prevalence in the age group 16-25. The prevalence fell slightly in older subjects.

5.4 Investigation C
Assessment of *H pylori* status following initial endoscopy

5.4.1 The test - ^{13}C-urea breath test

The aim of this test was to determine *H. pylori* status in patients who had recently undergone endoscopy, and note whether this status had been altered by the endoscopy.
These patients had been investigated at the time of endoscopy, looking for *H. pylori* in the mouth as well as stomach and duodenum, using the RUT, PCR and culture. After the endoscopy, patients were followed up for 6-12 weeks by breath test.

The principle was to determine whether there was any urease in the stomach by feeding the patient a meal containing urea labeled with ^{13}C. *H. pylori* possesses a very high urease activity, which splits ^{13}C-Urea solution into ammonia and carbon dioxide. The $^{13}CO_2$ expired pre- and post-urea can then be analysed.

$$^{13}CO(NH_2)_2 + H_2O \xrightarrow{\text{urease}} NH_3 + NH_3 + {}^{13}CO_2$$

Kits for the ^{13}C- urea breath test were purchased from the Bureau of Stable Isotope Analysis (BSIA Ltd Brentford, Middlesex, U.K).
A test kit consists of:

1. Test meal

The purpose of the test meal was to delay gastric emptying so as to allow enough time for the urease enzyme to act on the ^{13}C- urea molecule. 200 mls pure orange juice was used as the test meal.

2. Urea solution

This was prepared for use by adding 25 ml of water to the 100mg ^{13}C-urea dispensed in a 60 ml bottle.

3. Exetainer tubes

3 tubes for PRE-UREA breath; 3 for POST-UREA breath were labeled accordingly.

4. Sampling straw

5. Clerical forms

Consisting of analysis request forms, labels and patient's information notes.

5.4.2 The test procedure

I carried out the test, following the protocol suggested by the manufacturers. Subjects arrived at clinic, having fasted overnight. Fully informed consent was obtained after explaining the procedure. Age, sex and usage of drugs since the last endoscopy were noted. Patients who had had eradication therapy for *H. pylori* or were on antibiotic therapy for other reasons were not invited for the test. The first 100 patients to meet the criteria were tested.

At ZERO TIME

The subject was given the test meal of 200 ml pure orange juice.

At FIVE MINUTES

PRE-UREA breath samples were collected in triplicate by asking the subject to breathe normally down the straw into the bottom of the exetainer tubes until condensation appeared. The straw was then removed slowly and a screw cap applied immediately to the tube.

At TEN MINUTES

The subject drank the urea solution and then lay down on the couch on each side for two minutes and then rested for duration of test without food or drink.

At FORTY MINUTES

POST-UREA samples were collected in the same way as the pre-urea samples.

The tubes were labeled with the name and identification number of the subjects. Two pairs each of samples pre- and post-dose were placed into a plastic bag, placed in the pre-paid envelope and sent to BSIA on the day of the test. One set of pre- and one of post-urea samples were retained as insurance against loss or damage.
The samples were analysed by BSIA using mass spectrometry and results were usually ready, sealed and posted back to me within 3 working days. The envelope was not opened on receipt; all the results were stored and only recorded after the completion of all the tests.

5.4.3 Results:

The first 100 patients (out of 208) to return for the breath test were included in the analysis. 60/100 patients were *H. pylori* positive while 40/100 were negative at the endoscopic tests.
According to the breath test, of the 60 *H pylori*-positive at endoscopy, 39 remained positive while 21 became negative. Of the 40 negative patients, 31 remained negative while 9 had become positive (see table 14). These results, broken down into the various diagnoses are given in table 15.

The results of endoscopic diagnosis of patients who presented with dyspepsia (see table 16).

CHAPTER SIX

ANALYSIS OF RESULTS.

6. Analysis of results

6.1.1 The logistic regression analysis

Factors associated with *H pylori* infection and DU in patients attending endoscopy were analysed by logistic regression. There were 661 episodes of endoscopy involving 450 patients. In 302 of the episodes the patient was female, in 359 male. In 253 of the episodes the patient was a smoker, in 312 a non-smoker, and in the remaining 96 cases smoking status was not recorded.

208 patients were undergoing endoscopy for the first time in each study period. 161 had had one previous endoscopy, 93 had had 2, and then there was a diminishing number to 1 patient who had had 10 and one 11 previous endoscopies.

H pylori was negative in 193 episodes, positive in 209, and unknown (not tested) in 259. Information about anti-*H pylori* treatment was markedly incomplete, with details of such treatment immediately preceding or succeeding endoscopy being absent in about 480 episodes. Because of this, information about anti-*H pylori* treatment was excluded from the analysis.

Dependent variable *H pylori:*
The factors explored were gender, age, smoking, number of previous endoscopies and endoscopic diagnosis. Significant associations were obtained with gender ($p < 0.0001$; males much more likely to be *H pylori* positive than are females), number of endoscopies ($p < 0.0001$),

oesophagitis ($p < 0.0043$; *less* likely to be *H pylori* positive than subjects without oesophagitis)

Dependent variable DU:
Gender, age, *H pylori* status, smoking status and number of previous endoscopies were entered into the analysis. Significant factors favouring the presence of DU were age ($p < 0.001$), smoking ($p < 0.0001$), *H pylori* and number of endoscopies (see tables 4 and 5).

6.1.2 Endoscopic sterilisation.

No evidence of ineffectual sterilisation procedures was obtained. In all cases, the washings from the sterilised endoscopes failed to yield *H pylori* on PCR (see table 6).

6.2 Prevalence of *H pylori* in dyspeptic subjects

This investigation was carried out to determine the proportion of dyspeptic patients who were infected with *H pylori* at the time of undergoing endoscopic examination for their complaints. The diagnosis of DU as determined at endoscopy was also compared with the other endoscopic diagnoses for the relative frequency of *H pylori* infection. I studied 208 dyspeptic patients using the PCR technique which makes it possible to detect tiny amounts of *H. pylori* DNA. Several studies have used other diagnostic methods and gave varying prevalence reports. PCR is highly sensitive- approximately 100% in detecting hp in gastric biopsies; 96% in gastric juice; 60% in saliva (Van Zwet et al 1993).

Of the 208 patients, 114 were male, 94 female, and the peak age group was 46-55 years.

These were the results broken down into endoscopic categories; 90/208 (43%) had NUD i.e. no endoscopic abnormality was found; 37/208 or 18% had duodenal ulcer (DU); oesophagitis was diagnosed in 25/208 or 12%; 12/208 (6%) had gastric ulcer while 44/208 or 21% had miscellaneous diagnoses (Others).

The overall prevalence of *H pylori* in these patients was 114/208 or 55% as determined by the positive gastric biopsies using PCR technique.

The prevalence of *H pylori* in each of the diagnostic categories was 50/90 NUD; 32/37 DU; 7/25 Oeso; 9/12 GU; 16/44 Other (see table 17).

The prevalence obtained using the other biopsy techniques of RUT and culture were also investigated.

Using RUT as the criterion, the overall prevalence of *H pylori* infection was 75/208 or 36%. Using culture as the criterion, the overall prevalence was 34%. Using combined RUT/culture criterion, the overall prevalence was 86/208 or 41%.
Compared to PCR, the prevalence obtained using RUT and/or culture were consistently lower both in the overall group and the individual diagnostic groups (see tables 11 and 12).

I have also analysed the results of the factors influencing the prevalence rate of *H pylori* infection in this study. The information was obtained with a questionnaire which was completed before endoscopy. Positive *H pylori* status was based on the PCR results of the antral biopsies on all the dyspeptic patients. These factors are:

Age

Prevalence study stratified by age showed an increase in the prevalence rate of *H pylori* infection with age, reaching a peak in the 60s. There was a slight decline in the over-75 age group in this study.

Gender

H pylori appeared to be commoner in males than in females in the overall result, although this was not statistically significant. This was a reflection of the prevalence in the peptic ulcers group where there were more infected males than females. However, there was no difference in the prevalence in the NUD group.

Smoking

There was no apparent difference between smokers and non-smokers. 41/80 or 51% of smokers; and 73/128 or 57% of non-smokers were *H pylori*-positive.

Ethnic background

The group of patients recruited into this study predominantly live in metropolitan London. Prevalence rates of *H pylori* infection in the ethnic groups were: 84/164 or 52% Caucasians; 5/8 or 62% Africans; 16/26 or 69% Asians of Indian origin; 7/10 or 70% Asians of Chinese origin.

Length of history

The duration of symptoms of dyspepsia was analysed as shown in table 32. None of the patients in the DU category with symptom duration shorter than 6 months was *H pylori* positive. However, after 6 months, there was a 96% association with *H pylori.*

On the contrary, patients with NUD behaved differently. Under 6 months, 24 patients (53%) were *H pylori*-positive while 21 (47%) were *H pylori*-negative. Over 6 months, 20 (67%) patients were positive while 10 (33%) were negative.

The length of history is thus a highly significant factor in *H pylori* infection in DU patients. (χ^2, $p < 0.0001$)

Number of endoscopies

Further analysis of the relationship between the length of history and number of endoscopy is shown in tables 33 and 34. In the DU category, regression analysis showed a significant relationship between the duration of symptoms and the number of endoscopies (χ^2, $p < 0.0144$).

While the overall prevalence of *H pylori* in the dyspeptic group was 55%, there were considerable variations in the individual endoscopic groups.

Considering patients with DU only, the prevalence was found to be 86%. The peak age was 56-65 years where there was 100% association with *H pylori* infection. No duodenal ulcer was found in patients below the age of 26 in this study, out of a total of 7 with dyspepsia. In the 26-35 age group, out of 28 dyspeptic patients, there were only 2 with duodenal ulcer of which only 1 was associated with *H pylori*. Smoking was strongly associated with DU; there were 26/37 or 70% smokers compared to only 11/37 or 30% non-smokers with DU. On the contrary, there was no difference in the prevalence rates of *H pylori* in smokers (23/26 or 88%) and non-smokers (9/11 or 82%) in infected DU.

The prevalence of *H pylori* in patients with DU was compared with the prevalence in the NUD group. The overall prevalence in the latter was lower (56%) than in DU, but the trend showed a similar increase with age. There were more non-smokers (66/90 or 73%) than smokers (24/90 or 27%) in the NUD group although the prevalence of *H pylori* in NUD smokers and non-smokers appeared to be closer, 67% and 52% respectively.

The prevalence of *H pylori* in patients with oesophagitis (Oeso) was lower than in the other endoscopic groups. Of the 25 patients examined, only 7 or 28% tested positive.

Comparing DU with the miscellaneous group, the prevalence of *H pylori* in the miscellaneous group was lower at 36%. This was also lower than the overall prevalence of 55% (see table 17).

My results have also been analysed in the light of two previous studies done in this department:

1) Prevalence of *H pylori* in healthy controls (Tunio 1995).

2) Prevalence of *H pylori* in dyspeptic patients (Chandrakumaran 1991).

These were the results:

Tunio (1995) studied 100 healthy subjects (49 male, 51 female), using the ^{13}C- urea breath test to determine their *H pylori* status. The overall prevalence of *H pylori* was 39%, commoner in male than in female (χ^2, $p = 0.0003$). Age stratification showed a tendency to an increased prevalence with increasing age, rising to a peak at 65 years (see table 17). There was no statistically significant difference between smokers and non-smokers, although *H pylori* infection was commoner in non-smokers than in smokers (χ^2, $p = 0.068$).

Chandrakumaran (1991) studied 252 (100 male, 152 female) dyspeptic subjects using RUT, culture and histology, but not PCR. This group comprised 123 (49%) patients with NUD; 76 (30%) DU; 30 (12%) Oesophagitis; 23 (9%) with miscellaneous diagnoses including 5 with gastric ulcer. The overall prevalence of *H pylori* in the series was 63% and in each of the four diagnostic groups was 67% NUD; 70% DU; 30% Oesophagitis; 61% miscellaneous. In Chandrakumaran's study, the incidence of *H pylori* infection increased with age, with the peak age range of 56-65 years, and there was a decline after that. The mean age for *H pylori* positivity (54 years) was higher than the mean age for *H*

pylori negativity (40 years). More of the subjects in the DU group were smokers but there was no statistically significant difference between smokers and non-smokers in the *H pylori*-positive patients ($\chi^2 = 0.0780$, $p>0.4$).

In Chandrakumaran's study, the prevalence of *H pylori* infection in DU was surprisingly lower than what has been reported in many other studies. He had used RUT, culture and histology to determine the presence of *H pylori* infection and found that every patient who was positive by culture was also positive by histology. I have repeated this study using RUT, culture as well as PCR, obtaining a prevalence consistent with Chandrakumaran's study.

Final combined analysis in the age group in these studies were done since the studies were carried out in the same geographical area, (although, at different times), in the same hospital, using identical endoscopic methods and laboratory procedures (except for the PCR). Dyspeptic studies in the two series were compared with the control group (see table 21).

6.3 Detection of *H pylori* in the mouth, stomach and duodenum.

My aim was to investigate the upper gastrointestinal (GI) tract for the presence of *H pylori* infection. Because of the difficulty encountered by several researchers in detecting the microorganism from these GI sites, I have employed the PCR technique as well as the other conventional methods of culture and RUT (biopsies only).

6.3.1 Sensitivity and specificity of PCR methods for detecting *H pylori.*

The concentrations of each of the components of the PCR and the thermocycling parameters were determined and optimized by using known positive and negative controls as the target DNA.
The primers were found to be 100% sensitive and 100% specific; the known positive control of *H pylori* strain consistently produced a DNA fragment of the anticipated size. Similarly, none of the negative control strains of *E coli* and sterile water produced any bands. A magnesium chloride concentration of 2 mM gave the best yield; below this concentration, there was a decline in yield, showing as faint bands. Above this concentration especially at 6 mM, there was no improvement in the size of the bands obtained. Other reagents were used in concentrations which best reconciled economical running costs with maximum specificity and sensitivity as indicated by the band obtained.
1 ng of the known *H pylori* DNA used as the experimental positive control template was diluted eightfold, serially, with the expected products with each dilution. Although superior yield were obtained at dilution up to fourfold, at no time did aberrant bands appear.

In 10 PCR-negative samples (2 each of saliva, plaque, gastric juice, biopsy and duodenal aspirate), the addition of known *H pylori*- positive solution gave positive amplified products. As explained earlier, this was to determine any false-negative results which may occur if there were inhibitors in the clinical samples.

A two-step cycling programme gave comparable yields to the three-step PCR programme, but had the advantage of considerable savings in the total run time.

The PCR-amplified products were analysed by agarose gel electrophoresis and photographed under ultra-violet light. Samples were recorded positive when a band of 109 base pair could be detected on the agarose gel.

Gastric antral biopsy specimens were used as the basis of comparison between PCR technique and the endoscopic tests in this study. 208 PCR and RUT; and 180 cultural tests were performed.

H pylori was identified in 55%, 36% and 34% of patients according to PCR, RUT and Culture criteria respectively. PCR correctly detected *H pylori* in 74 of 75 RUT cases and all (61 cases) of culture, thus recording one false-negative result based on RUT criterion, and, no false-negative result based on the criterion of culture technique. The single false-negative sample was also negative on culture.

Of the 119 patients with negative culture, 115 were negative on RUT and 80 on PCR. 4 and 31 more patients were positive on RUT and PCR respectively.

6.3.2 Oral site.

Specimens were tested by PCR and culture. None of the saliva or the tongue and oropharyngeal swabs was *H pylori*- positive by any test. When known positive strains of *H pylori* were added to two saliva specimens, they became positive by PCR.
15 of 208 dental plaque specimens tested positive by PCR. Only 2 of these were further positive by culture. The sex distribution was equal (8 male; 7 female), age between 25 to 79 years (mean age 55 years; peak age group 56-65 years). Eleven (11) of these patients were Caucasians and 4 were Asians of Indian origin. There was no difference in their smoking pattern; 8 were smokers while 7 were non-smokers. None of these patients was edentulous or had any obvious periodontal pathology. The causes of their dyspepsia were found as follows: NUD 5; DU 8; GU 1; Gastritis 1. 7 patients had undergone previous endoscopic investigations, while 8 patients attended for the first time. The 2 patients who were also positive by culture had NUD and were aged 35 and 61 years respectively.

6.3.3 Gastroduodenal sites

No sample of gastric or duodenal juice was positive by culture. However, 36(17%) samples of gastric juice and 6 of 50(12%) samples of duodenal juice were positive by PCR.

The antral biopsies were *H pylori*- positive as follows; 114 (55%) positive by PCR; 75(36%) positive by RUT; 61 of 180(34%) were positive by culture.

6.4 The relationship between oral and gastrointestinal sites.

Overall, only 6 patients had *H pylori* in the three sites, viz., oral (dental plaque), stomach and duodenum. These 6 patients plus 9 more were positive in two sites, viz, oral and stomach. Restriction endonuclease digestion of the amplified PCR products from these 15 patients showed that 13 of the dental plaque isolates had identical restriction digest patterns to those of the organisms in the stomach. In the case of the remaining two patients, the patterns were slightly different. On the other hand, only 4 of the 15 patients had a similar pattern in all three sites - plaque, stomach and duodenum. In the other 11 duodenal samples, 2 had different patterns while 9 yielded no identifiable PCR products.

6.5 Variability of *H pylori* status after endoscopy.

A preliminary analysis of the record data from the endoscopy clinic indicated that there might be a link between the presence of *H pylori* in the mouth and the stomach through the passage of an endoscope. Logistic regression had suggested number of endoscopies was a factor in *H pylori* positivity. Was it possible that *H pylori*-negative subjects were becoming infected during endoscopy? One possibility was inadequate sterilization, but evidence suggested not. Another was that the passing of the endoscope mechanically carried organisms down from the oral cavity.

In this study, 100 patients who had previously undergone routine endoscopy were invited for the non-invasive ^{13}C- Urea Breath Test, to determine their *H pylori* status.

The patients (57 male; 43 female) were diagnosed at endoscopy as NUD (46), DU (14), GU (5), Oesophagitis (16), Others (19). At endoscopy, the result showed that 42 patients were *H pylori*- positive while 58 were negative. The corresponding breath test results were 49 positive and 51 negative, indicating conversion rates of 26% (from -ve to +ve) and 19% (from +ve to -ve status). This showed a large two-directional variation in *H pylori* status over the course of 6-12 weeks.

Further analysis showed the effect of the individual diagnosis on this variation in *H pylori* status. Of the 14 DU patients, 9 were positive and 5 negative at endoscopy. All the 9 positive patients remained positive at breath test i.e. 0% conversion from +ve to -ve. On the other hand, 2 of the 5 negative patients became positive at breath test i.e 40% conversion from -ve to +ve. In a similar manner, 17 patients with NUD tested positive and 29 negative at endoscopy. 13/17 remained positive (i.e. 25% +ve to -ve); while 21/29 remained negative (i.e. 28% -ve to +ve). This individual analysis is shown in table 15.

Changes affect all diagnoses, although *H pylori* status was stable in the majority of patients, NUD patients were less stable than DU and GU patients.

CHAPTER SEVEN

DISCUSSION

7.1 Discussion of methods

7.1.1 Tests of *H pylori*

7.1.1.1 Subjects

The majority of patients I recruited into this study were referred by their general practitioner for investigation of their dyspeptic symptoms. Overall, 208 patients were enrolled - 114 male; 94 female, with peak age cohort 56-65 years. These patients live locally in this metropolitan area of London.

With regard to individual symptoms, these included epigastric pain/discomfort; nausea; heartburn; excessive eructations; abdominal distension in that order of frequency.

Some of these patients had initially been treated by their general practitioners. This is of course due to widespread treatment of 'ulcer-like' symptoms with H_2-antagonists. Patients on drugs such as proton pump inhibitors and antibiotics may pose special problems. Bacterial density may diminish with PPI (Logan et al 1995, Dickey et al 1996), while antibiotics will eradicate the organism. Thus, *H. pylori* cannot be adequately identified by a single clinical test. Discontinuation of PPI before embarking on biopsy methods is recommended, since as little as one week time lag may be sufficient for return of bacterial density in the stomach.

7.1.1.2 Techniques

The main questionnaire used for recording patient's details was carefully designed bearing in mind the variable factors associated with peptic ulcer disease. A record was made of age, gender, ethnic origin, smoking history, drug history, previous endoscopy and length of history. The past medical history, drug history, use of anti-*H pylori* treatment, NSAID, proton pump inhibitors, any anti-ulcer treatment, either in the past or present, were noted. The endoscopic diagnosis at previous endoscopy and the treatment action taken were also documented.

When collecting saliva, the patient was asked to simply spit or dribble into a sterile container, until an adequate volume had been collected. Plenty of time was allowed for this, as some patients found it a difficult, if not embarrassing, task. An oropharyngeal and tongue swab was taken by repeatedly stroking these areas with the swab stick. The collection of the dental plaque was performed under the direct supervision of a dentist. The microorganisms of the plaque are different from those of the tartar. Using a sterile periodontal curette, both supra- and sub-gingival plaques were collected by a gentle upward scrape against the tooth surface. The deeper layer contains tartar which is harder than the plaque and does not truly represents the oral microbial population. The oral specimens were collected before performing the endoscopy in order to avoid contamination with gastric contents while withdrawing the scope.

7.1.1.2a Biopsy technique

Three antral biopsy specimens from non-adjacent sites were obtained for further tests for the presence of *H. pylori*. Large-channel biopsy forceps were used requiring separate passes for optimal tissue sampling. Sampling errors may arise when the bacterial density is low (Morris et al). Similarly, when the distribution of the organism in the stomach is patchy, false negative results would be found.

Successful sampling depends on the site and number of biopsies, and perhaps a significantly large (in terms of surface area) biopsy specimen using large-channel biopsy forceps. In many studies, the prepyloric antrum has been the preferred site of biopsy. The sensitivity of a single biopsy from the angle of the stomach is reported as 96-97%, the sensitivity of two separate biopsies as 100%. Currently, there is a new multiple-bite forceps, providing up to four biopsies with only a single pass (thus saving time and effort). However, these biopsies are significantly smaller than those provided by the large-channel forceps, and there are no data to support the opinion that four small biopsies will provide as good a diagnostic yield as two or three large biopsies.

7.1.1.2b Tests

Culture is a standard diagnostic technique for *H. pylori* infection. False negative results could arise from errors in obtaining the specimen, storage and transport. It is specific and has sensitivity greater than 95%. Viability of *H pylori* during transport is best guaranteed in sterile saline (up to 6 hours). Saline is readily available, simple and in some studies, *H. pylori* can survive in it for up to 24 hours. Each specimen collected was labelled and transferred to the microbiology laboratory and

inoculated into Colombia agar with 5% horse blood and 10 μg/ml amphotericin B culture medium within three hours of collection. In 28 patients, the samples could not be cultured in time and were therefore excluded from this part of the test, but were tested by the PCR method.

Histological assessment provides a reliable method for detection of *H pylori* based on its unique appearance in tissue sections. Histology was done with the routine staining technique. Histology was only needed in this study for confirming the diagnosis based on the initial endoscopic assessment. Where histology was done (in 60 patients), culture , RUT and PCR were also done. There was 100% concordance between the positive histology result and positive culture and PCR results but not RUT, in which there was one isolated positive result, when the other methods were negative. None of the *H pylori*-negative DU submitted to histology was positive by histology.

The biopsy urease test detects the presence of preformed enzyme urease produced by *H pylori* in the biopsy specimen by a colour (pH) change due to the ammonia liberated. The CLOtest which was the first of these tests to be developed is available commercially (Delta West Ltd., Perth, Australia). This test has been modified and improved. The urease test used in this experiment was the modification by Vaira et al which was originally developed in this department and has been in use since 1988. This solution consists of modified urea broth with an increased urea concentration of 6% in order to improve its sensitivity. A biopsy specimen is placed in the urea broth, which was prepared by Dr. John Holton, and has been tested and used in this department since 1988. This is a modified urea broth originally prepared by Christensen and later, McNulty and Wise. The sensitivity attained at 1 hour by the original 2% Christensen's urea broth was 61%. This also contained nutrients which

allow the growth of urease-producing organisms, which can lead to false positive results. Our modification is cheap, easy to prepare and perform, and has improved speed of result and sensitivity at 1 hour (89%).

The speed of result is dependent on the concentration of the substrate. The ideal concentration of substrate in an enzyme reaction is usually 50-100 times the Km* value of the enzyme, i.e. a measure of affinity of an enzyme for its substrate. *H. pylori* enzyme urease has a Km* value of 0.32 g/litre or 8 mM. The ideal concentration of urea is 16-32 g/litre or 1.6-3.2%. At even higher concentration (6-10%), the speed of the reaction is enhanced and the test results are rapidly available.

Only a broth volume of 500 µl or less is needed and it is kept at room temperature. False positive results are very rare and the specificity is almost 100%. This is because contaminating organisms are usually present in low numbers compared with *H. pylori*, and no other organism produces urease with such high affinity.

RUT, culture and histology are commonly used in making a clinical diagnosis of *H. pylori*. Each has its advantages as enumerated in the previous chapters. However, because of the nature of the specimens in this study, culture can be applied to all the specimens while histology and RUT can only be done on tissue biopsy specimens. Culture alone would not give an accurate detection rate. Several studies have reported the difficulty in growing this microorganism especially from saliva, dental plaque and gastric juice. In view of this, amplification by PCR, which makes it possible to detect tiny amounts of DNA from *H. pylori* was employed to test all the specimens.

7.1.1.2c DNA amplification technique

PCR is highly sensitive- approximately 100% in detecting *H pylori* in gastric biopsies; 96% in gastric juice; 60% in saliva. As a result of this, small levels of DNA contamination can lead to enormous amplification of wrong products. Contamination can result from previous PCR amplification reactions, samples with high levels of DNA, positive control templates and the use of plastic tubes and pipettes.

AmpErase UNG was used in these experiments to ensure that the products of the previous PCR amplifications could not be reamplified to produce false positive results.

Measures taken to avoid contamination:
A dedicated working area was set up and physically separate benches assigned to the different PCR stages. Each work station had its own supply of pipettes, racks, tubes and other disposable items. At the start of every experiment, all work surfaces were cleaned and then dried off with industrial methylated spirit, including the inside of the microfuge, rotor, tube racks, etc. A separate, clean laboratory coat and gloves were used at different stages of the experiment. All the tips and pipettes were purchased sterile and siliconized, while other PCR tubes were sterilised by autoclaving or filtration. Only precision microlitre pipettes with high-density polyethylene-plugged tips were used. Once each aliquot had been pipetted, sterile oil was added to each tube to prevent evaporation losses, and the tubes were kept closed and on ice, until loaded onto the cycler.

Optimal reagents and samples, and precautions:

I spent the first period of the experiments determining the optimal reagent concentration and quality test control, before testing clinical material. Strains of *E. coli*, a urease-negative organisms and sterile water were used as negative controls. Positive controls of known *H pylori* strains were kindly provided by Dr. John Holton. The threshold of detection was determined through serial dilution of the *H pylori* strain. Dilution up to eight-fold still gave detectable *H pylori* bands.

Ten (10) PCR-negative clinical samples were tested in order to exclude sub-optimal conditions. The problem of false-negative results was eliminated by adding known *H pylori* positive cells to 10 PCR- negative samples. This was also to verify whether inhibitors or other sub-optimal conditions were present in the PCR-negative samples. The total volume per reaction was 100 μL, the volume of water was adjusted as long as the total volume of the reaction remains constant. The primers, templates and magnesium chloride concentrations were varied individually until clear bands of the anticipated sizes were obtained.

7.1.3 ^{13}C-urea breath test

7.1.3a Patient preparation

The first suitable 100 patients that returned after 6-12 weeks of the initial endoscopy were tested using the ^{13}C-urea breath test. This was partly due to the fact that it is expensive to test all the 208 patients that underwent endoscopy; and partly due to logistics in successfully re-recruiting these patients. Patients were rejected if they had undergone eradication therapy during this period.

7.1.3b Choice of test

The nature of this study required that the patient returned for follow-up monitoring of *H. pylori* status following the initial endoscopy. The ideal way was to repeat the endoscopic test in the same manner as before. However, endoscopy is expensive, uncomfortable and carries a small but finite risk. It is not ethically justified especially when the benefit to the patient is only of secondary importance. Breath tests, according to the literature, equally accurately assess *H pylori* status and are simple, non-invasive, more convenient and preferred especially when the extra information yielded by an endoscopy is not needed. The ^{13}C-urea breath test can be repeated as often as necessary and has no side effects.

Histology, culture and RUT are all biopsy-based tests, and due to patchy distribution of *H. pylori* in the stomach, are liable to observer variation and sampling error. They are also invasive, whereas the ^{13}C- urea breath test is not.

The serology test (ELISE), is also non-invasive and widely-used. It has a disadvantage in that it is less predictive. The serum antibody titres are found in the serum for a long time, maybe up to a year after the eradication of *H pylori*, whereas the breath test is only positive in current infection.

The ^{13}C is a stable, naturally-occurring isotope. It does not decay and has an infinite half life (unlike ^{14}C isotope). It is therefore safe in children and in pregnancy, and can be sent by post!

7.2 DISCUSSION OF RESULTS

7.2.1 Prevalence of *H pylori* in dyspeptic patients in a metropolitan area.

In this study, I have investigated the microorganism *H pylori* in relation to NUD, DU, Oesophagitis, GU and the miscellaneous group. I have also found the prevalence in the individual age group and the apparent effects of age, gender, ethnic background, length of history and smoking on *H pylori* infection in the group studied.

No normal controls were used in this study because this had been investigated in this department in 1993. The previous investigation was carried out by Tunio and consisted of 100 healthy, asymptomatic subjects examined by a non-invasive ^{13}C-urea breath test.
Tunio studied one hundred healthy control volunteers who were predominantly medical and nursing students. The overall prevalence of *H pylori* infection in healthy asymptomatic subjects was 39%. This was stratified by age and there was a progressive increase in the prevalence of *H pylori* with the age groups, although there was some tendency towards a fall in those aged over 65 years. There was no statistically significant difference between smokers and non-smokers, although there appeared to be a tendency for smoking to protect against *H pylori* (χ^2, p= 0.068). The prevalence of *H pylori* was commoner in men than in women (χ^2, p=0.0003) see table 13.

I have studied *H pylori* as an aetiological factor in relation to the aetiology of DU, in patients attending an endoscopy unit for the

investigation of dyspepsia. The results of my study showed that the overall prevalence of *H pylori* in dyspeptics was 55%. In a similar investigation in healthy controls (Tunio 1995), a prevalence of 39% was reported. Other studies, from developed countries, have reported prevalence of 32% (Dooley et al 1989), 36% (Wilhemsen et al 1994), 41% (Vaira et al 1994), 52% (Graham et al 1991a). On the other hand, studies from developing countries have reported higher prevalence; 80-90% of populations of Vietnam, Algeria and Ivory Coast (Megraud et al 1989), 100% (Majmudar et al 1990).

One striking point from my study, is that the prevalence of *H pylori* in dyspeptics increases with age, reaching a peak in the 56-65 age group and tending to fall afterwards. On the average, *H pylori* was higher above, than below the age of 55 years. In the control study as well as other studies in the literature, similar increases with age were observed (Jones et al 1986, Dooley 1988, Dill 1990, Meyer 1991, Peterson 1991, Graham 1991b, Asaka 1992, Lin 1993, Smoak 1994, Vaira 1994). The reported peak age group in the control study was 46-55 years(68.4%), as was the case in some other studies (Vaira 1994, Mossi 1993, Meyer 1991). The tendency for the infection rate to fall has also being observed in other studies (Mossi 1993).

I have found no statistically significant difference between the sexes. This has been supported by several studies which have shown that there is no difference in the prevalence of *H pylori* in men and women (Graham 1991a, Megraud 1989, Wilhelmsen 1994, Forman 1993, Rocha 1992, Mossi 1993). However, some studies have reported a higher prevalence among female smokers (Fich 1993).

Smoking habits of my dyspeptic group have no significant association with *H pylori* status. This was also the case in several other studies that compared the prevalence of *H pylori* in smokers and non-smokers (Graham 1991a, Forman 1993, Vaira 1994). It is perhaps noteworthy that in the control study by Tunio(1995), whereas there was no statistically significant difference between smokers and non-smokers, there was a trend towards lower prevalence among non-smokers.

The effect of ethnic origin on *H pylori* status in my group showed a lower prevalence of the organism in Caucasians than in the other ethnic groups. However, this difference was not statistically significant. Reported studies of the effect of ethnic groups on prevalence have been inconsistent; a lower incidence of *H pylori* in the Aborigines of Australia compared with Caucasians. Patients in my study live in metropolitan London, the majority being descendants of immigrants.

The prevalence of *H pylori* in NUD, DU, Oesophagitis, GU and Others, in our endoscopy clinic were 56%, 86%, 28%, 75% and 36% respectively (by all tests). In the control group it was 39%. It thus appears, that most patients attending an endoscopy unit for the investigation of dyspepsia are more likely to have the infection than normal controls. However, patients with oesophagitis had a lesser infection rate than normal subjects. The explanation for this observation may be because of the direct effect of acid in the oesophagitis patients due to reflux which destroys the organism.

The prevalence of *H pylori* in DU in my series was 86% with PCR, and 69% without PCR. Chandrakumaran had studied similar patients in this department and found a prevalence rate of 70%, using the conventional RUT, histology and culture techniques. In other clinics where DU

patients were specifically studied, prevalence close to 100% have been reported, and this has been one of the strongest argument in implicating *H pylori* as the principal aetiological factor in DU. By using PCR technique, which has been shown to be more sensitive than the other conventional tests, my finding of only 86% prevalence of *H pylori* in DU has further strengthened confidence in the results of the initial investigation by Chandrakumaran. This finding is also in line with other studies in which rates of between 50 and 90 % had been reported (50% Uyub et al, 53% Spiliadis 1991, 76% Hsu 1992, 67.7% Kachintorn 1993).

This difference cannot simply be explained on the basis of age, although some studies have observed marked effect of age on the prevalence of *H pylori* (Jones 1986, Dooly 1988, Dill 1990, Meyer 1991, Peterson 1991, Graham 1991b, Asaka 1992, Lin 1993, Smoak 1994, Vaira 1994). This showed increasing prevalence of infection with age. 100% prevalence rate was only found in the age range 56-65 years based on the PCR tests. DU is not predominantly a disease of the elderly (see table 22). The effect may be as a result of their length of history rather than age.

There was a statistically significant difference between smokers and non-smokers in DU. This difference was not noticed in *H pylori* infected DU subjects. The significance of this is that while smoking is an important factor in DU disease itself, it does not appear to influence *H pylori* infection. This observation was also corroborated by Lindell et al (1991), who found a lower prevalence of *H pylori* among smokers than non-smokers. The lower prevalence of *H pylori* in smokers was thought to be due to a direct toxic effect of nicotine on colonization with *H pylori*. In Chandrakumaran's study of dyspeptics, and in the healthy

control (Tunio 1995), smoking was not a significant factor in *H pylori* infection.

There was no effect of gender on the prevalence of *H pylori* in either the dyspeptic group or in DU patients. This was also the case in the control group. There were more male than female patients with DU in my study, but there was no significant difference in the prevalence of infection.

As mentioned earlier, the logistic regression analysis of factors associated with *H pylori* infection suggested that the infection might be related to the number of endoscopies a patient had undergone. Although the exact mechanism of *H pylori* infection is not known, the oral has been quoted as the likely route of the infection. It is known that the organism is often present in saliva (Krajden 1989, Ferguson 1993) and in dental tartar (Majmudar 1990, Krajden 1989, Desai 1991, Shames 1989, Oshowo et al 1998). This oral site may constitute a reservoir of organism that the endoscope might carry down to the stomach.

Because many patients underwent endoscopy more than once, there is potential confounding effect of multiple observations on the same individual. Logistic regression analysis was therefore based on a) first endoscopy ever, i.e., patients who had never undergone endoscopy before being seen at The Middlesex Hospital; and b) patients undergoing endoscopy for the first time in the series under review, although they had previously undergone endoscopy either at The Middlesex or elsewhere. The results show that there is no link between *H pylori* and the number of endoscopies undergone because those who had never undergone endoscopy had no higher a prevalence of infection than those who had had one or more endoscopies.

In view of the finding that the relationship between number of endoscopies and infection with *H pylori* was spurious, and that the former variable appeared to correlate with the duration of symptoms, I re-examined the data to seek any link between the duration of symptoms and prevalence of the infection. The evidence is in table 32, none of 5 patients with symptoms of a duration less than six months was *H pylori* positive; 32 of 33 (92%) patients with symptoms that had been present for six months to 5 years were *H pylori* positive.

With this in mind, I compared the pattern of infection in DU and NUD patients in relations to the number of endoscopies and the length of history of dyspepsia (see table 33, 34). None of the 5 DU patients whose symptom duration was less than 6 months was *H pylori*-positive, while over 6 months, 96% of the DU patients were *H pylori*-positive. In the NUD group, the results were 53% and 67% respectively. The length of history is statistically highly significant in *H pylori* infection in DU patients. ($p < 0.0001$). Analysis of variance (ANOVA) showed a significant relationship between the length of history and the number of endoscopies ($p < 0.0144$).

7.2.3 Detection of *H pylori* from gastrointestinal sites.

I wanted to know whether *H pylori* colonises the mouth en route to its site of election in the pyloric antrum. In this study, there was never any evidence of *H pylori* in the oral site except in the plaque, where it was

present in 15 out of 208 (or 7%) of all the dyspeptic patients using PCR, and only in 2(1%) using culture. These results suggested that colonisation of the mouth by *H pylori* is a rather unusual event, and that it does not occur except in plaque. Similar results were obtained by other studies (Megraud 3.2%, Ishihara 18%, Krajden 3.4%). Several studies have shown rare reports of culturing *H pylori* from the mouth (Bernander et al (0%)). However, in India where there is high prevalence of *H pylori*, a study reported culturing *H pylori* from 100% of 40 volunteers. In a recent review of studies of oral carriage of *H pylori* by Madiner et al(1997), the recovery rate of oral *H pylori* ranges from 0 to 100%.

The problem in culturing *H pylori* from the dental plaque may be related to the mode of collection, whether the plaque was supra- or sub-gingival, from specific site or simply pooled samples. It has been reported that the organism was more likely to be residing in the sub- rather than supra-gingival plaque, particularly where the gingival pocket was > 4 mm in depth (Cao CF et al 1997). The complex forms of the numerous organisms in the plaque may prevent colonization of *H pylori*. Studies have identified the numerical dominance of gram-negative organisms in deep periodontal pockets and root canals. The microbiology of dental plaque is complex and includes numerous fragile and fastidious forms, which makes it difficult to culture.

The technique of PCR seemed to be more sensitive than culture in detecting *H pylori*. This may mean that culture gives the true picture of present colonisation while the PCR in addition also gives evidence of past colonisation, in that it is picking up fragments of dead organisms.

The question as to whether the oral cavity in general and dental plaque specifically is a potential reservoir and possible sanctuary for *H pylori* infection, is controversial. Cases of re-infection following successful eradication treatment have been attributed to oral sites. Some studies have suggested that the role of dental plaque as a permanent reservoir is self-evident. Other investigators, however, have argued against this notion. The results from the present study showed 7% oral colonisation. The detection of *H pylori* in dental plaque in this study suggests that *H pylori* colonisation is not restricted to the gastric mucosa. However, the failure to detect *H pylori* from other sites in the oral cavity, together with the low prevalence of *H pylori* in the dental plaque would tend to suggest that the oral cavity is not a permanent reservoir. Indeed, it is more likely that *H pylori* is a transient rather than a permanent resident in the oral cavity. The reason for its presence in the oral cavity may be as a result of gastric reflux, although the possibility of direct oral-oral or oral-faecal transmission cannot be ruled out. Evidence from a study of 436 individuals tested for *H pylori* by the serum antibody method whose hepatitis A status was also analysed, strongly suggested that the faecal oral route is not the method of spread for *H pylori* (Webb et al).

Whatever the reasons, this study did not support the notion that the oral cavity is a reservoir or sanctuary which may be responsible for reinoculation of the stomach after eradication therapy for *H pylori* infection.

My investigation of the gastrointestinal tract showed that the stomach is a commoner site of colonisation. In the gastric biopsy samples, 116 (55%) dyspeptic patients were positive. It also suggested that if *H pylori* is present in the stomach, it may also be detected in the mouth in about 14% by PCR (much less by culture). Of 92 patients with no trace of the

organism in the stomach, none showed evidence of *H pylori* in the mouth.

Detection rate of *H pylori* from gastric juice and duodenal aspirate by culture was zero. This demonstrates that the organism cannot live in acid although it is present in the stomach, but when it gets into the gastric juice it dies. *H pylori* was detected by PCR in gastric juice in 36 patients(17%), and 6 of 50 (12%) patients in whom duodenal aspirate was obtained. Both sets of patients also had positive antral biopsy specimens. The detection of *H pylori* from these gastrointestinal secretions was important for transmission potential in *H pylori* infections. So far, the exact route of transmission of *H pylori* is not known. It has been suggested that the route of transmission are oral-oral and oral-faecal. The concept that the transmission is faecal-oral is adhered to widely, as the organism has been detected from drinking water.

The results demonstrating the presence of *H pylori* from oral, gastric and duodenal sites have given support to the concept of the oral route. Gastrointestinal secretions harbour *H pylori* and may play a significant role in the transmission, especially in vomiting and diarrhoeal diseases.

In the present study, when H pylori was present in the oral cavity, it was always present in the gastric sites, and occasionally in the duodenum.

Similar investigation also revealed that if present in the stomach it may be detected in the mouth in about 14% (by PCR), and if not present in the stomach it was never found in the oral cavity.
DNA extracted from *H pylori* from different sites in the same patients and digested with Hae III, provided visual bands after electrophoresis

under ultraviolet light. Only 6 patients had *H pylori* detected from the duodenum. These and 9 others from gastric and dental plaque were digested to determine if they were epidemiologically linked. 13 patients had dental digestion pattern indistinguishable from those of the corresponding biopsy. 4 isolates from the duodenum similarly had same digestion patterns as in the dental plaque and antral biopsy. These similarities provided evidence of a linear transmission relationship between these sites.

From the epidemiological point of view, growth from sites other than the stomach are of interest but rarely reported.

7.2.4 *H pylori* status in dyspeptic patients following endoscopy.

The logistic regression analysis of factors associated with *H pylori* infection and DU had been analysed, suggesting a relationship with endoscopy. With this hypothesis in mind, *H pylori* status at endoscopy was determined and compared after endoscopy, to check if the passage of the endoscope had in any way, affected the individual status.

There was a considerable conversion in either direction over the course of 6-12 weeks, despite absence of anti-*H pylori* eradication treatment. This result is perhaps more interesting when analysed in relation to the disease entities. None of the DU group converted from positive to negative, while NUD formed 50% of the total converted. Changes affected all diagnoses, whereas *H pylori* status was stable in the majority

of patients, NUD patients were much more affected. On the contrary, DU and GU patients were much less affected. This variability of *H pylori* status following endoscopy would indicate that the 'non-ulcer' population is much less stable than we have previously believed.
The size of the two-directional variation in *H pylori* status from this study is surprising. The endoscopic tests of culture and RUT were used in the analysis to forestall any suggestions that the variability was due to dead fragments picked up by PCR.

Conversion from positive to negative (19%) suggested that there was no active infection or that the organism had been eliminated following endoscopy. None of the patients tested were on any antibiotics between the period of their endoscopy and breath test. Also, this conversion could not be explained on the basis of passage of an endoscope.

Conversion from negative to positive (26%) would suggest acquisition of *H pylori* infection following the procedure. As endoscopy was the only identifiable factor before and after the tests, one could attribute this difference to endoscopy, which would also support the results of the logistic regression analysis. The mechanism could be by direct inoculation from an infected (dirty) endoscope, or by direct mechanical transmission from the oral cavity during the passage of the endoscope. It could also be due to mechanical damage to the stomach during the procedure, which was later invaded by *H pylori.* However, evidence from this study did not support any of the above possibilities.

The result of the investigation of oral cavity showed scanty colonisation by *H pylori* (7%). None of the patients with positive conversion after the endoscopy showed any evidence of *H pylori* in their mouth before the endoscopy. Moreover, tests of endoscopy sterilisation technique did

not show any evidence of contamination or fault in the sterilisation procedure. My conclusion, therefore, is that endoscopy itself has no effect on the conversion rates in either direction.

Is *H pylori* a cause of DU?

I set out to investigate the relationship between *H pylori* and DU based on the previous studies from this department, and the general hypothesis that *H pylori* is the immediate cause of DU. I have assessed the extent to which a causal relationship between *H pylori* and DU can be demonstrated, through several investigations.

The starting point was to repeat Chandrakumaran and Vaira's investigation who have found a low prevalence of *H pylori* in DU (70%), using the standard procedures of culture, histology and RUT. When the very sensitive method, PCR, was used in my experiments, the prevalence rose to 86%, still short of the 90-100% level reported by many other centres. But my results using the standard procedures of culture, histology and RUT, compared well with Chandrakumaran's.

I have evaluated the methodology involved in Chandrakumaran's investigation and mine. Sources of error could be from the specimen handling and the laboratory methods employed in the processing. If the low prevalence in Chandrakumaran's was due to faulty technology, then mine must be similar! My evidence about concordance between RUT, histology and culture, where all were available, except for 1 positive by RUT only, suggests that the laboratory procedure was satisfactory. So it was likely that there were some *H pylori* -negative DU. This is supported by the PCR which only gave 86%, not 100%.

Therefore, if the low prevalence were technical errors, they must have arisen from handling of samples. Procedures involved in the collection, transport and storage were correctly applied and strongly adhered to as discussed in the previous chapters. This is evident from the prevalence results obtained from NUD patients in my study, compared with Tunio's healthy controls. This comparison between my prevalence for NUD with Tunio's normal healthy controls, show they were of the same order or at least not lower. So the handling of NUD samples must have been okay. If those samples were okay, surely the handling of all the samples (including duodenal ulcer), must have been okay.

The next major issue arose from the retrospective study of the factors associated with DU and *H pylori* infection in patients attending for an endoscopy. This was analysed by the Logistic regression method. Arising from this study was that association between *H pylori* and DU in dyspeptic patients was linked to the number of endoscopies. This was further substantiated by the evidence from the investigation of the oral cavity, stomach and gastrointestinal sites which confirmed colonisation. Restriction endonuclease also confirmed the presence of the same strain in these different sites. We also know from this GI study that when *H pylori* was absent in the stomach, it was never found in the mouth. This evidence supports the oral transmission of *H pylori*. It also raises the important issue of the endoscopic transfer of the organism from the oral cavity to GI sites. This may be from a contaminated or poorly sterilized instruments, or merely by mechanical dislodgment of the organisms to a more distal site. It may also be that endoscopes, by sheer trauma to the gastric mucosa, increases the susceptibility to *H pylori* colonization. Since the influence of endoscopy might clearly have been related to a lack of adequate sterilisation of endoscopes, the endoscopic sterilisation procedure was tested for evidence of the organism by PCR. It was clear

that although endoscopes can be contaminated by *H pylori* organisms, sterilisation procedures were found to be adequate.

Further evidence was sought from the investigation with ^{13}C-urea breath test, which followed up *H pylori* status of dyspeptic patients for 6-12 weeks. This did not show any nett change in *H pylori* status following endoscopy. If number of endoscopies a patient has undergone is associated with *H pylori* positivity, then the mechanism is not just by the mechanical effect. When the breath test was further analysed, it appeared that patients with DU were less likely to shed their *H pylori* status over a period of time, than patients with no endoscopic lesions at all. It would appear that the organism was 'glued' to a lesion (DU), and less liable to be dislodged than when there was no lesion! This finding was consistent with other studies which showed a significant reduction in *H pylori* colonization after surgical treatment and ulcer healing (Steer et al 1988). Also Kunzle et al (1997) investigated the prevalence of *H pylori* after various acid-reducing surgery for DU. They concluded that DU recurrence was not subordinated to *H pylori* following surgery. Similarly, various studies have shown that the number of recurrent ulcer after vagotomy increases with time and is limited to patients with incomplete vagotomy, and that *H pylori* may promote the development of recurrent DU only in incomplete vagotomy cases (Peetsalu et al 1998; Martin et al 1995; Svoboda et al 1991).

The preliminary results of the logistic regression analysis of the factors associated with *H pylori* suggested number of endoscopies. Restricting the clinic series to (a) first endoscopy ever, and (b) first endoscopy in the series only, demonstrated that it was not number of endoscopies. It is difficult to be certain how these should be interpreted. A reason for the spurious correlation of number of endoscopies with prevalence was

sought. The answer seems to be the duration of symptoms at the time of the first endoscopy. There were only five patients whose symptoms had lasted less than 6 months: they were all *H pylori* negative. By contrast, 11 of the 12 whose symptoms had lasted six months to a year, and 11 of the 12 whose symptoms had lasted between one and 5 years, were *H pylori* positive.

Five is a small number on which to base a conclusion that *H pylori* is not present in DU patients early in their disease and is therefore not the cause of their disease. However, the difference between 5 out of 5 before six months, and 22 out of 24 after six months, is highly significant (Fisher's exact p=0.0004). This finding would indicate a lower prevalence of *H pylori* in DU patients with a less than six months, compared with a greater than six months, duration of symptoms. Emanating from the logistic regression analysis, the length of history is statistically highly significant in *H pylori* infection in DU patients. ($p < 0.0001$) . Patients whose duration of symptoms were 6 months or less, were mainly *H pylori-* negative. There is also a correlation between the length of history and number of endoscopy.

Analysis of variance showed a significant relationship between the duration of symptom and the number of endoscopies ($p < 0.0144$).

I compared the pattern of infection in DU and NUD patients in relations to the number of endoscopy and the length of history of dyspepsia. *None of the 5 DU patients whose symptom duration was less than 6 months was H pylori-positive. On the other hand, 96% of the DU patients who had dyspeptic symptoms >6 months, were H pylori-positive. In the NUD group, the results were 53% and 67% respectively.*

The mechanism whereby the organism reached the ulcer does not appear to be direct, such as the passage of an endoscope, dislodging the organism from its oral reservoir to the stomach. The length of history, rather than the number of endoscopies done, appears to determine *H pylori* status in DU patients but not NUD patients. What then is the explanation for this association between length of history and the presence of infection in DU but not in NUD patients?

H pylori is reported to be present in 50% and almost 100% of people in developed and developing countries respectively. This makes the organism the commonest chronic bacterial infection worldwide. Sometimes, it is pathogenic and at most times, it simply exists in the gastrointestinal tract of apparently healthy people.

My finding that patients with DU of longer duration are more likely to have *H pylori* infection requires further discussion as it suggested that the ulcer preceded the infection. If the infection preceded the ulcer, one would expect to find the organism in the ulcer patient at all times. It may be that the organism was destroyed at the early stage by the effect of the gastric acid but later, as a result of adaptation, the organism was able to re-colonize the ulcer. However, several studies have associated DU with other factors, notably smoking and excessive acid secretions. The initial breach of the mucosa may possibly be due to the effect of acid, the ulcer remained unhealed, and then attracted the organism. While the organism remains in the ulcer, the ulcer would not heal. This would also explain why ulcers heal after eradication therapy. This latter explanation has also been supported by studies which showed a significant reduction in *H pylori* colonisation after surgical treatment of

duodenal ulcer. In previous decades, persistent peptic ulceration was often treated surgically either by vagotomy or by partial gastrectomy.

In the recent systematic review of 36 studies of surgical treatment for peptic ulceration, the prevalence of *H pylori* is said to remain high after vagotomy (83%) but falls to about 50% after partial gastrectomy, in which the ulcer had been resected. Proximal gastric vagotomy as well as truncal vagotomy with pyloroplasty do not influence the incidence rate of *H pylori* gastritis.
Partial gastrectomy removes the antrum and so would be expected to substantially to reduce *H pylori* ; after all, it is the antrum that is the site of predilection. Vagotomy reduces gastric acid secretion without removing the antrum and therefore would be expected to have no effect on *H pylori,* or even to increase *H pylori.*

The question of whether *H pylori* causes DU has been assessed in these investigations, using the principles of cause and effect. *H pylori* infection of the stomach does not seem to cause DU, but the presence of DU may predispose to infection, with the organism.

CHAPTER EIGHT

CONCLUSIONS

8. Conclusions.

1) Using PCR, 14% of patients with DU presenting at The Middlesex Hospital for routine endoscopy are *H pylori* negative. Tests not involving PCR however, showed much more.

2) The logistic regression analysis showed that the most important correlate with *H pylori* infection was the number of previous endoscopies that had been performed on the patient, the most important correlate with DU was the fact that the patient was a smoker.

3) The link between *H pylori* infection and endoscopy is unlikely to be due to a straight infection by the endoscope itself or by the endoscope carrying the organism down from the mouth.

4) PCR is the most sensitive test for *H pylori* and can be used on various specimens. However, it also picks up dead or fragmented *H pylori* organisms as demonstrated by lack of the ability to culture living organisms from gastric or duodenal aspirates which are PCR positive.

5) *H pylori* colonises the mouth but only in 7% of those with infected stomach. Oral colonization may be transient rather than permanent.

6) None of the oral samples was positive by any test for *H pylori* except in the dental plaque.

7) When *H pylori* was not present in the stomach, it was never found in the mouth.

8) If in stomach, *H pylori* can be found in 14% in the mouth by PCR (much less by culture)

9) If in the mouth, *H pylori* is always same strain as in the stomach.

10) The conversion rates in both directions following endoscopy would suggest that *H pylori* status in 'non-ulcer' population is considerably more labile than we have previously believed.

11) *H pylori* is not present in patients who have had symptoms of duodenal ulcer for less than six months. The significance of this is far from clear: either *H pylori* is not the principal cause of DU or it is present in the early case but is difficult to detect.

Suggested further work should be on:
i) The relationship between infection in acute and, chronic DU.
ii) Prospective study on the pattern of infection in dyspeptic patients at intervals, perhaps 6- or 12- monthly.
iii) The relationship between gastric acid secretion and *H pylori* in DU patients.

9. LIST OF ABBREVIATIONS.

Helicobacter pylori	- *H pylori*, HP.
Duodenal ulcer	- DU
Non Ulcer Dyspepsia	- NUD
Oesophagitis	- Oeso.
Rapid Urease Test	- RUT
Polymerase Chain Reaction	- PCR
^{13}C- Urea Breath Test	- ^{13}C-UBT
Gastrointestinal	- GI
Thermus aquaticus	- Taq
Deoxyribonucleotide triphosphate	- dNTP
Uracil N-glycosylase	- UNG

LIST OF TABLES

TABLE 1: The genus *Helicobacter*

SPECIES	**NATURAL HOST**
H pylori	Human
H felis	Cat, Dog
H mustelae	Ferret
H nemestrinae	Primates
H acinonyx	Cheetah
H heilmanii *(Gastrospirillium hominis)*	Cat, Dog, Pig
H fennelliae	Human rectal swab
H cinaedi	Human rectal swab
H muridarum	

Table 2: Prevalence of chronic gastritis and seropositivity for *H pylori* with age.

Age-range (yrs)	%positive for hp antibodies	%with chronic gastritis
0-9	<2	<10
18-25	18	10-20
26-35	30	20-38
36-45	46	36-40
46-55	59	40-58
>55	55	60-65

Table 3: Rates of *H pylori* infection in patients with gastrointestinal pathologies.

Diagnosis	no. of studies	no. of patients	*H pylori* %positive Mean	(range)
No gastritis	9	230	9	(0-20)
Active chronic gastritis	9	401	88	(64-95)
Duodenal ulcer	8	246	85	(75-100)
Gastric ulcer	8	178	64	(35-86)

Table 4: Factors associated with *H pylori.*

Factor	**p value**
gender(male)	<0.0001
no of endoscopies	<0.0001
oeso.(negative)	<0.0043
NUD	<0.0236
DU	<0.0156

Table 5: Factors associated with DU

Factor	**p value**
age	<0.001
smoking	<0.0001
H pylori	<0.0138
no of endoscopies	<0.0122

Table 6: Test of endoscopic sterilisation procedure.

	Pre-sterilisation status		**Post-sterilisation status**
Patient	RUT	PCR	PCR
1	+	+	-
2	+	-	-
3	+	+	-
4	+	+	-
5	+	-	-
6	+	+	-
7	+	-	-
8	+	+	-
9	+	+	-
10	+	+	-
11	+	+	-
12	+	-	-
13	+	-	-
14	+	+	-
15	+	-	-
16	+	-	-
17	+	+	-
18	+	+	-
19	+	-	-
20	+	+	-
21	-	-	-
22	-	-	-
23	-	-	-

Table 7: Occurrence of *H pylori* in dyspeptic patients using different diagnostic methods.

	PCR (n=208)	**Culture** (n=180)	**RUT** (n=208)
Saliva	0	0	-
Swab	0	0	-
Plaque	15(7%)	2(1%)	-
Gastric juice	36(17%)	-	-
Gastric biopsy	114(55%)	61(34%)	75(36%)
Duodenal aspirate	6/50(12%)	-	-

Table 8: Successful isolation of *H pylori* from dental plaque using culture technique (1).

Patient's name	A.K
age	34
gender	female
smoking	none
previous ulcer disease	none
previous endoscopy	none
symptom (main)	eructations
symptom duration	12 months
previous HP treatment	none
medication	antacids only
endoscopic diagnosis	NUD
other +ve test results:	biopsy(RUT, culture and PCR) +ve gastric juice(PCR) +ve
^{13}C-UBT(after endoscopy)	+ve

Table 9: Successful isolation of *H pylori* from dental plaque using culture technique (2).

name	D.N
age	61
gender	female
smoking	none
previous ulcer disease	none
previous endoscopy	none
symptom (main)	indigestion
symptom duration	>6 months
previous HP treatment	none
medication	none
endoscopic diagnosis	NUD
other +ve test results	biopsy(RUT, culture, PCR) +ve gastric juice(PCR) +ve
^{13}C-UBT(after endoscopy)	+ve

Table 10: Successful detection of *H pylori* from dental plaque using PCR technique.

age	sex	symptom	smoking	diagnosis	pre-HP	pre-endo	E
47	m	pain	yes	DU	nil	2	C
69	m	pain	yes	DU	nil	2	C
38	m	indigestion	yes	DU	?	1	AS
55	m	pain	no	DU	nil	2	C
48	f	vomiting	yes	gastritis	nil	1	C
61	m	pain	yes	DU	nil	1	C
56	f	indigestion	no	NUD	nil	1	AS
61	f	indigestion	no	NUD	nil	1	C
60	f	indigestion	no	NUD	nil	1	AS
79	m	pain	no	DU	nil	2	C
25	f	indigestion	no	NUD	nil	1	AS
36	f	pain/indiges	yes	GU	nil	2	C
67	m	indigestion	yes	DU	yes	5	C
62	f	indigestion	yes	NUD	nil	1	C
65	m	pain	no	DU	nil	2	C

E= ethnic origin; C= Caucasian; AS= Asian(Indian)

Table 11: Test of *H pylori*- Culture versus RUT.

	RUT +ve	**RUT -ve**
Culture +ve (61)	54	7
culture -ve (119)	4	115

The sensitivity of RUT (versus culture) from my result was therefore,

--------- 54/61 = 89%

The specificity was --------- 115/119 = 98%

Table 12: Test of *H pylori*- Culture versus PCR.

	PCR +ve	**PCR -ve**
Culture +ve (61)	61	0
Culture -ve (119)	31	88

i.e. Culture is 100% sensitive but 88/119 or 74% compared with PCR.

Table 13: Prevalence of *H. pylori* in healthy controls, subdivided by age (Adapted from Tunio 1994)

Age(yrs)	**Total**	**Positive**	**%Positive**
16-25	19	03	15.8
26-35	14	04	28.6
36-45	19	05	26.3
46-55	19	13	68.4
56-65	21	12	57.1
>66	08	02	25.0

Table 14: Conversion rates of *H pylori* 6-12 weeks after endoscopy.

Before	**After**	**Conversion**
60 +ve	39 +ve; 21-ve	+ --> - = 21/60 (35%)
40 -ve	31 -ve; 9+ve	- --> + = 9/31 (23%)

Table 15: Details of 100 patients who attended for the breath test.

a) Table showing nett conversion from positive to negative

Endosc. Diagnosis	Number (n = 100)	Endosc. Hp +ve	Breath Hp +ve	Breath Hp -ve	Nett change (+ve to -ve)
NUD	46	17	13	4	-4
DU	**14**	**9**	**9**	**0**	**0**
GU	5	3	2	1	-1
Oeso	16	4	2	2	-2
Others	19	9	8	1	-1
TOTAL	100	42	34	8	-8

Conversion rate from positive to negative of 8/42 or 19%.

b) Table showing nett conversion from negative to positive.

Endosc. Diagnosis	Number (n = 100)	Endosc. Hp -ve	Breath Hp -ve	Breath Hp +ve	Nett change (-ve to +ve)
NUD	46	29	22	7	+7
DU	14	5	3	2	+2
GU	5	2	2	0	0
Oeso	16	12	8	4	+4
Others	19	10	8	2	+2
TOTAL	100	58	38	15	+15

Conversion rate from negative to positive of 15/58 or 26%.

Table 16: Endoscopic diagnosis of patients who presented with dyspepsia. (Patients presenting as emergency or complications were not included in the study).

GROUP	n = 208	%
NUD	**90**	**43**
DU	**37**	**18**
OESOPHAGITIS	**25**	**12**
GU	**12**	**6**
Others	**44**	**21**

Table 17: Frequency of *H. pylori* infection in endoscopic group.

GROUP	HP +ve	%
NUD	50	56%
DU	32	86%
OESOPHAGITIS	7	28%
GU	9	75%
Others	16	36%
TOTAL	114	55%

Table 18: ENDOSCOPIC GROUP STRATIFIED ACCORDING TO AGE.

AGE	NUD	DU	OESO	GU	Others	Total
< 26	4	0	0	1	2	**7**
26-35	19	2	4	0	3	**28**
36-45	17	11	4	3	4	**39**
46-55	18	8	6	1	14	**47**
56-65	17	8	4	1	11	**41**
66-75	9	5	4	2	5	**25**
> 75	6	3	3	4	5	**21**
Total	**90**	**37**	**25**	**12**	**44**	**208**

Table 19: PREVALENCE OF *H. PYLORI* STRATIFIED ACCORDING TO AGE USING PCR TECHNIQUE.

Age	**NUD**	**DU**	**OESO**	**GU**	**OTHERS**	**%**
<26	2	0	0	1	0	42%
26-35	10	1	2	0	2	54%
36-45	8	9	1	2	2	56%
46-55	5	7	3	0	3	38%
56-65	13	8	0	1	4	63%
66-75	8	5	1	2	3	76%
>75	4	2	0	3	2	52%
TOTAL	50	32	7	9	16	55%

Table 20: PREVALENCE OF *H. PYLORI* STRATIFIED ACCORDING TO AGE USING CULTURE/RUT TECHNIQUES.

Age	**NUD**	**DU**	**OESO**	**GU**	**OTHERS**	**n**
<26	2	0	0	1	0	3
26-35	8	1	2	0	2	13
36-45	7	9	0	1	2	19
46-55	3	6	2	0	4	15
56-65	8	7	0	0	3	18
66-75	5	3	1	0	3	12
>75	2	0	0	3	0	5
TOTAL	35	26	6	5	14	86

Table 21: Prevalence of *H pylori* stratified according to age - combined results 1991 (Chandrakumara) and 1996 (Oshowo).

Age	**NUD**	**DU**	**OESO**	**OTHERS**	**Total**
<26	4	2	0	1	7
26-35	16	8	2	2	28
36-45	22	22	3	6	53
46-55	33	24	6	7	70
56-65	34	19	2	12	67
66-75	18	8	2	5	33
>75	6	2	1	6	15
TOTAL	133	85	16	39	273

Table 22: % Prevalence of *H pylori* in NUD and DU in age groups.

Age	**NUD**	**DU**
<26	2/4(50%)	0
26-35	10/19(53%)	1/2(50%)
36-45	8/17(47%)	9/11(82%)
46-55	5/18(28%)	7/8(88%)
56-65	13/17(76%)	8/8(100%)
66-75	8/9(89%)	5/5(100%)
>75	4/6(67%)	2/3(67%)
TOTAL	50/90(56%)	32/37(86%)

Table 23: Proportion of smokers in DU and NUD.

Group	Smokers	Non-smokers	p value
DU	26	11	$\chi^2 = 0.0032$
NUD	24	66	

Table 24: Proportion of *H pylori*-positive smokers in DU and NUD.

	Smokers	**non-smoker**
DU	23(88%)	9(82%)
NUD	16(67%)	34(52%)

Table 25: Prevalence of *H pylori* in dyspeptic patients- pattern of sex distribution.

	MALE	Hp+ve	FEMALE	Hp+ve
NUD	40	22	52	28
DU	30	27	7	5
OESO	12	4	13	3
GU	4	4	8	5
TOTAL	114	70	94	44

Table 26: Comparison of diagnostic tests- Culture, RUT and PCR.

	Culture +ve	RUT +ve	PCR +ve	n
NUD	22(28%)	32(36%)	50(56%)	90
DU	22(65%)	22(59%)	32(86%)	37
OESO	6(29%)	4(16%)	7(28%)	25
GU	3(43%)	5(42%)	9(75%)	12
OTHERS	8(21%)	12(27%)	16(36%)	44
TOTAL	61(34%)	75(36%)	114(55%)	208

Table 27: Comparison of diagnostic tests- PCR vs. RUT/Culture

	PCR +ve	RUT/Culture +ve	TOTAL
NUD	50(56%)	35(39%)	90
DU	32(86%)	26(70%)	37
OESO	7(28%)	6(24%)	25
GU	9(75%)	5(42%)	12
OTHERS	16(36%)	14(32%)	44
TOTAL	114(55%)	86(41%)	208

Table 28: Comparison of diagnostic tests- RUT vs. Culture/PCR

	RUT +ve	**Culture/ PCR +ve**
NUD	32	50
DU	22	32
OESO	4	7
GU	5	9
TOTAL	75	114

Table 29: Prevalence of *H pylori* in 208 dyspeptic patients using RUT, culture and PCR criteria. (Culture was not available in all patients).

	PCR	RUT	Culture	RUT/Culture
NUD	50	32	22/80	35
DU	32	22	22/34	26
Oeso	7	4	6/21	6
GU	9	5	3/7	5
Others	17	12	8/31	14

Table 30: The possible effect of gender, smoking, as demonstrated in this study.

	NUD	DU	Oeso	GU	Others
male sex	55%	90%	33%	100%	61%
female sex	53%	71%	23%	63%	47%
% smokers	67%	88%			

Table 31: ANOVA Table showing Regression Summary - number of endoscopy vs. length of history.

	DF	Sum of squares	Mean square	F-Value	P-Value
Regression	1	107.937	107.937	57.185	<0.0001
Residual	35	66.063	1.888		
Total	36	174.000			

Total count 36

Number missing 0

Standard Error 0.006

Confidence intervals

95% Lower 0.031

95% Upper 0.054

Table 32: Length of history and *H pylori* infection in DU and NUD patients.

Duration	DU (non-PCR)	DU (PCR)	NUD (non-PCR)	NUD (PCR)
< 1 month	**0/2**	**0/2**	**3/8**	**3/8**
1-3months	**0/1**	**0/1**	**2/14**	**7/14**
3-6months	**0/2**	**0/2**	**9/23**	**14/23**
6-12months	**11/12**	**12/12**	**4/7**	**5/7**
1-5years	**8/9**	**9/9**	**1/4**	**4/4**
>5years	**5/9**	**9/9**	**3/3**	**3/3**

Table 33: Length of history and number of endoscopies - DU

Duration	1	2	3	4	5
<1month	**2**	-	-	-	-
1-3months	**1**	-	-	-	-
3-6months	**1**	**1**	-	-	-
6-12months	**4**	**6**	**1**	**1**	-
1-5years	**3**	**5**	**1**	-	-
>5years	**1**	**4**	**3**	-	**1**

Table 34: Length of history and no of endoscopies- NUD

Duration	0	1	2	3	4	5	6
<1month	**8**	**2**	-	-	-	-	-
1-3months	**8**	**3**	-	-	-	-	-
3-6months	**12**	**7**	-	-	-	-	-
6-12months	**5**	**1**	**1**	-	-	-	-
1-5years	**1**	**3**	**1**	-	-	-	**1**
>5years	**2**	-	-	-	-	-	**1**

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