The intragastric bacterial flora in relation to gastric malignancy : methodological and clinical studies

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The intragastric bacterial flora in relation to gastric malignancy

methodological and clinical studies

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*Aan: mijn ouders
Karel*

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Chapter I

Introduction and aims of the study

Introduction

The *Helicobacter pylori* (*H.pylori*) is associated with gastric and duodenal ulceration and with an increased risk of gastric cancer and gastric mucosa-associated lymphoid tissue (MALT) lymphoma. However, the exact role of *H.pylori* in these diseases is not yet clear and other factors, like host and environmental factors, may also be involved. Moreover, other bacteria, in this study called the non-*H.pylori* bacterial flora, can also be present in the stomach depending on the intragastric pH. Their presence is associated with several clinical lesions in which *H.pylori* can also be found.

Helicobacter pylori

The first report about the presence of spiral bacteria in the stomach of animals results from Bizzozero in 1893¹. The first study in humans was performed by Doenges² who found spirochaetes in the gastric glands of 43% of human stomachs examined in routine autopsies. In 1983, Warren and Marshall³ described the presence of unidentified curved bacilli on gastric epithelium in active chronic gastritis and were the first to culture the organism. The bacterium was identified as *Campylobacter pyloridis*⁴ and was later given the name *Helicobacter pylori*.

Microbiology

H.pylori is a Gram-negative S-shaped rod measuring 0.5 x 3.0 μm with four to six sheathed flagella attached to one pole⁵⁻⁷. The bacterium is highly motile and possesses enzymes including oxidase, catalase, alkaline phosphatase, and a high urease activity^{5,8,9} but does not reduce nitrate, hydrolyse hippurate, or metabolize glucose^{5,6}. *H.pylori* grows under micro-aerophilic conditions and produces small (1 mm) translucent colonies within 3 to 4 days at 37°C^{5,10}. This fastidiously growing microorganism requires fresh media made with fresh blood or serum and maintenance of an adequate humidity throughout the incubation^{6,11,12}. Bacterial cells in older cultures or exposed to oxygen may give rise to coccoid forms^{6,9}. The viability of these coccoid forms is still under debate.

The *H.pylori* urease has a low Michaelis constant permitting the enzyme to operate with a high efficiency at low urea concentrations¹³. By its extreme urease activity, *H.pylori* can convert urea into ammonium and bicarbonate⁷. Ammonia is an H^+ acceptor leading to a local rise in pH¹⁴, thus creating an alkaline micro-environment that protects *H.pylori* from the intragastric acidity.

H. pylori has been adapted to the gastric micro-environment by the potent urease activity, its micro-aerophilism, the spiral shape and high motility. These last two characteristics enable the bacterium to move through the gastric mucus towards the gastric epithelium⁹, probably attracted by chemotaxins and/or growth factors¹⁵.

Epidemiology

H. pylori infection occurs worldwide. The prevalence of infection varies between geographical regions¹⁶. The incidence of *H. pylori* has been declining over time. *H. pylori* infection starts in childhood^{17,18}. The prevalence increases with age^{17,19,20} from about 10%-20% in the age of 18-29 years to 50%-70% in the age of 60-69 years^{21,22}. In developing countries the *H. pylori* prevalences are very high²³⁻²⁵ and a majority of children become infected^{16,26}. In adults in industrialized countries, the incidence of new *H. pylori* infections is 0.5-1.0% per year while in developing countries the incidence varies between 3% and 10% per year¹⁸. *H. pylori* infection usually persists for many years and the spontaneous eradication rate appears to be low^{16,27}.

An increased risk for *H. pylori* infection is associated with non-white race and low socio-economic status^{17-20,22,28}. No association could be found with use of NSAIDs, alcohol or current smoking^{19,21}. The source of *H. pylori* infection has not been found yet but evidence for person-to-person transmission results from studies showing a high *H. pylori* prevalence in institutions^{29,30}, clustering of *H. pylori* within families^{17,31-33}, and linkage of *H. pylori* with conditions associated with residential crowding and poor hygiene^{17,20}. Furthermore, some studies found evidence for a role of waterborne transmission²⁶, and also a correlation of *H. pylori* infection with the consumption of uncooked vegetables has been reported²⁴.

Two routes for person-to-person transmission have been described: the faecal-oral and the oral-oral route. Findings of *H. pylori* in stool^{34,35} and in dental plaque or saliva³⁶⁻³⁸ support these routes. Person-to-person transmission is also demonstrated by a high *H. pylori* prevalence in gastroenterologists and endoscopists, especially in those who do not wear gloves^{19,39-41}. *H. pylori* may spread from contaminated instruments or speci-mens¹⁹.

Helicobacter pylori-associated gastritis

H. pylori is strongly associated with the presence of gastritis^{3,42-44}. The bacteria are only rarely found in the histologically normal mucosa and are almost always accompanied by active chronic gastritis characterized by a neutrophil infiltrate⁴⁵⁻⁴⁸. *H. pylori* is associated with type B antral gastritis which subsequently may spread out to the corpus⁴⁹.

A causal role for *H. pylori* in gastritis is supported by two studies of healthy

volunteers showing an acute antral inflammation and *H.pylori* colonization after ingestion of *H.pylori*^{49,50}. Furthermore, eradication of *H.pylori* results in an improvement or disappearance of the gastritis⁵¹⁻⁵³.

H.pylori will colonize the "normal" gastric mucosa and cause gastritis and will not preferably colonize an inflamed gastric mucosa as the prevalence of *H.pylori* is low in patients with gastritis associated with pernicious anaemia^{54,55}, eosinophilic gastritis⁵⁶ and Ménétrier's disease⁵⁶.

On long term, chronic *H.pylori*-associated gastritis can progress towards atrophic gastritis and intestinal metaplasia^{48,57,58}. With increasing mucosal atrophy and intestinal metaplasia, the colonization of *H.pylori* in the stomach decreases^{59,60}.

Peptic ulcer disease

H.pylori is associated with both duodenal and gastric ulcers. *H.pylori* prevalences range from 78%-100% in patients with duodenal ulcer disease^{42,61-65} and from 60%-100% in patients with gastric ulcer disease^{62,63,66,67}.

Nomura et al.⁶⁸ showed after a follow up period of 20 years that *H.pylori* positive subjects are at higher risk to develop peptic ulcer disease than *H.pylori* negative controls. In cross-sectional and follow-up studies, peptic ulcer disease was found in 2.8%-20% of *H.pylori* positive subjects^{21,69,70}. A higher prevalence was found for duodenal than for gastric ulcer disease. Duodenal ulcer patients are generally associated with antral-predominant *H.pylori* colonization and gastritis, a high intragastric acidity and the presence of gastric metaplasia in the duodenal bulb. Gastric ulcer patients are often found to be older with a more wide-spread *H.pylori* colonization and gastritis in both antrum and corpus, a decreased intragastric acidity and higher prevalences of atrophic gastritis than in duodenal ulcer patients⁷¹⁻⁷³. Recurrence of peptic ulcers has been prevented by eradication of *H.pylori* in both duodenal^{65,74-76} and gastric ulcer patients^{75,77,78}.

In general, acquisition of *H.pylori* is already mentioned to occur in childhood^{17, 18}. The development of a gastric ulcer appears to be especially related to an infection with *H.pylori* acquired at a young age while duodenal ulceration will develop from a *H.pylori* infection acquired at a later age^{79,80}.

A majority of subjects infected with *H.pylori* will not develop a peptic ulcer. Mechanisms in peptic ulcer pathogenesis and differences in pathogenesis of duodenal or gastric ulcer formation are not completely understood. Differences in host response, *H.pylori* strains, and environmental factors may play a role in the differential development of peptic ulcer disease of infected subjects. Environmental factors include diet, smoking and stress. Host response may differ with regard to the parietal cell mass, basal acid secretory capacity, gastrin levels, and mucus resistance⁷². A great genetic diversity in *H.pylori* strains has been shown⁸¹⁻⁸³.

Recently, the presence of *vacA* and *cagA* positive strains has gained much interest. The *vacA* gene encodes a vacuolizing cytotoxic protein. Almost all strains appear to possess the *vacA* gene which is only expressed in the presence of the cytotoxin-associated gene (*cagA*)⁸⁴. Several studies report an association between the presence of peptic ulcer disease and a *cagA* positive strain^{85,86}.

Gastric cancer

Two types of gastric cancer can be distinguished according to the classification by Laurén⁸⁷: the intestinal and the diffuse type. Although the incidence of gastric cancer is declining over time, it is still associated with a high mortality⁸⁸⁻⁹⁰. The decline is attributable to a decrease of tumours located in the distal stomach, while the incidence of cancer in the gastric cardia increases^{88,91}. The intestinal type is more often found in the distal stomach while the diffuse type develops throughout the stomach but especially in the gastric cardia⁹².

There is a wide geographical variation in the incidence of gastric cancer with high incidences in blacks and males⁹⁰⁻⁹³. Furthermore gastric cancer has been found to cluster within families and is associated with a low socio-economic status^{90,92,93}. These epidemiological findings of gastric cancer resemble those for the prevalence of *H.pylori*. Histological⁹⁴⁻⁹⁷ and serological studies⁹⁸⁻¹⁰² find an association of *H.pylori* with gastric cancer. Serological studies indicate a three-to-sixfold higher risk for *H.pylori* positive subjects to develop gastric cancer⁹⁸⁻¹⁰⁰. High odds ratios are especially found in young *H.pylori* positive subjects below 40 years of age^{103,104}.

The mode in which *H.pylori* contributes to the development of gastric cancer is still unknown. *H.pylori* associated gastritis may progress towards gastric mucosal atrophy and intestinal metaplasia which are known to be possible precancerous lesions, especially for the intestinal type of gastric cancer¹⁰⁵⁻¹⁰⁷. *H.pylori* may mainly play an initiating role in the histological cascade towards gastric cancer by causing a chronic gastritis, as subsequently *H.pylori* colonization is known to decrease in atrophic gastritis and intestinal metaplasia, and is even absent in the gastric tumour tissue itself. Although some studies report *H.pylori* to be more often present in the intestinal type of gastric cancer⁹⁵⁻⁹⁷, others find similar *H.pylori* prevalences in both the intestinal and the diffuse type¹⁰⁸⁻¹¹⁰.

Although some epidemiological studies find a similar distribution of *H.pylori* and gastric cancer¹¹¹, others find high *H.pylori* prevalences in gastric cancer patients in countries with low gastric cancer incidences¹¹². Recently, neither Kuipers et al.¹¹³ nor Webb et al.¹¹⁴ could support a role of *H.pylori* in gastric carcinogenesis.

Besides *H.pylori*, other factors have to be involved in gastric cancer: the

presence of nitrite and N-nitroso compounds in diet, low intake of vitamin C and E, and a high salt intake are associated with an increased risk of gastric cancer^{90,92,93}. Furthermore clinical conditions such as partial gastrectomy^{115,116}, achlorhydric atrophic gastritis with or without pernicious anaemia¹¹⁷⁻¹²¹, intestinal metaplasia¹⁰⁵, and use of H₂-receptor antagonists¹²² are associated with an increased risk of gastric cancer.

Primary gastric lymphoma

Primary gastric lymphoma account for 1-5% of all gastric malignancies¹²³. Normally lymphoid tissue is not found in the stomach but it is reported to be acquired in response to a *H.pylori* infection^{124,125}. The presence of lymphoid follicles and aggregates is found to correlate with the degree and activity of antral gastritis and the density of *H.pylori* colonization¹²⁵. In a clinical case study, Carlson et al.¹²⁶ followed a patient with *H.pylori*-associated gastritis that progressed to lymphoid hyperplasia and subsequently to the development of a monoclonal B-cell lymphoma. The presence of acquired mucosa-associated lymphoid tissue (MALT) may precede to the development of primary gastric lymphoma.

A serological nested case-control study by Parsonnet et al.¹²⁷ showed that patients with primary gastric lymphoma are more likely to have evidence of a previous *H.pylori* infection (odds ratio 6.3). An association is also found between the incidence of non-Hodgkin's lymphoma and the rate of *H.pylori* infection in an epidemiological study by Doglioni et al.¹²⁸. A high prevalence of *H.pylori* (80%-92%) has been found by histology in non-tumorous gastric mucosa of patients with primary gastric lymphoma of MALT origin^{123,124} and eradication of *H.pylori* has been shown to lead to a regression of the lymphoma¹²⁹⁻¹³¹. However, also lower *H.pylori* prevalences in gastric lymphoma (about 50%) are reported by others^{132,133}.

Pathogenesis

H.pylori is primarily associated with gastric epithelium, including areas of gastric metaplasia. The bacterium is found in the gastric mucus as well as in close proximity of the epithelial cells, mainly near the tight-junctions^{15,134-137}.

H.pylori is able to colonize the gastric mucosa by the extreme urease activity: *H.pylori* is surrounded by an alkaline cloud which protects the bacterium from the gastric acid^{13,15,137}. Urease plays an important role in infection as urease-negative *H.pylori* strains are not able to induce gastritis in gnotobiotic piglets¹³⁸. Furthermore, urea can be used as a nitrogen source^{13,137} and urease elicits an immune response¹³⁹.

In the mucus layer, the pH is near to neutral¹³⁵. *H.pylori* is adapted to the viscous milieu of the mucus by the high motility mediated by the flagella, the spiral

shape^{15,135} and the production of protease which degrades mucin molecules^{15,137}.

H.pylori can adhere to the gastric epithelial cells via non-specific factors like surface charges and hydrophobic interactions and more specific via lectins and adhesins¹⁵. Ultrastructural studies have shown the presence of adhesion pedestals between *H.pylori* and gastric mucosal cells¹⁴⁰. Adhesion results in depletion of microvilli and distortion of the cytoskeleton^{15,135,137}.

The initial response to *H.pylori* infection is accompanied by a period of achlorhydria¹⁴¹. *H.pylori* produces a protein inhibitor of gastric acid secretion¹³⁷. Also higher basal- and meal-stimulated gastrin values are reported in *H.pylori* positive DU patients¹⁴²⁻¹⁴⁴.

H.pylori elicits a local and systemic immune response: inflammatory cells migrate into the epithelium of the infected mucosa^{15,137} and IgG, IgA and IgM antibodies against *H.pylori* can be detected both in the gastric mucosa¹⁴⁵ and in the blood^{146,147}. The catalase activity protects *H.pylori* against toxic effects of reactive oxygen metabolites released during the inflammatory process¹⁴⁸.

H.pylori can induce tissue damage by the production of several factors that damage the gastric mucosal integrity^{13,137,139,149}. *H.pylori* can degrade the mucosal integrity by lipase and phospholipase activity¹³⁷, by inhibition of the binding of laminin¹⁵⁰, and by induction of pepsinogen secretion^{137,149}. Furthermore, ammonia is cytotoxic, especially by ammonium hydroxide¹³⁹ and certain *H.pylori* strains exert a vacuolating activity (vacA) which is associated with the presence of the cytotoxin-associated gene (cagA)⁸⁴. This cytotoxic activity is found in 50%-60% of *H.pylori* strains^{149,151}, and shows a higher prevalence in *H.pylori* positive duodenal ulcer patients compared to those without peptic ulcer disease^{86, 152,153}. Patients infected with a cagA positive strain, are at higher risk to develop atrophic gastritis and intestinal metaplasia¹⁵⁴ and a high cagA prevalence is found in gastric cancer patients¹⁵⁵. Finally, an increased oxidative DNA damage is shown in the *H.pylori*-infected human gastric mucosa¹⁵⁶ and *H.pylori* and its immune response result in an increased proliferation of the gastric epithelial cells¹⁵⁷.

Eradication therapy for *Helicobacter pylori*

After eradication of *H.pylori*, gastritis diminishes or resolves and recurrence of both gastric and duodenal ulcers markedly decrease. Furthermore a regression of gastric MALT-lymphoma has been demonstrated after *H.pylori* eradication therapy.

Good results with more than 80% eradication¹⁵⁸ are obtained with a triple therapy of bismuth, metronidazole and amoxycillin or tetracycline, but side effects are frequently reported and there is no good compliance of the patients¹⁵⁹.

The incidence of side-effects is lower using a two-week dual therapy consisting of the proton-pump inhibitor omeprazole in combination with amoxycillin or

clarithromycin¹⁶⁰. However, eradication rates are generally lower and show a wide variation^{159,161}. In a meta-analysis, eradication rates range from 53% to 72% with different antibiotics used¹⁶¹.

Better results are obtained using omeprazole with a combination of two antibiotics out of amoxycillin, clarithromycin or metronidazole¹⁶². Combining several studies, Tytgat¹⁵⁸ reports an eradication percentage of 87.5% using clarithromycin and metronidazole and 95.0% using clarithromycin and amoxycillin. A success rate of 85% to 90% was obtained by only one week treatment¹⁶³.

A recently introduced and very effective therapy is the quadruple therapy consisting of omeprazole, bismuth, metronidazole and tetracyclin for one week¹⁶⁴.

The results of the eradication regimens depend on the compliance of the patients and the antibiotic susceptibility of *H.pylori*. A penicillin-allergy has been reported in 5%-10% of the population which can therefore not use amoxycillin¹⁶⁵. Metronidazole resistant strains are reported in 10%-20% of patients in developed countries and up to 80% in developing countries¹⁵⁹. Metronidazole resistance can be overcome in vivo when given in combination with omeprazole¹⁶⁶. Also resistance against clarithromycin^{167,168} and tetracycline^{169,170} have been described.

Non-*Helicobacter pylori* intragastric bacterial flora

Role of intragastric acidity

The parietal cells are located over the entire wall of the body and fundus of the stomach except parts of the lesser curvature, and secrete hydrochloric acid and intrinsic factor^{171,172}. Gastric acid plays a role in the digestion of food by the conversion of pepsinogen into the active proteolytic enzyme pepsin, and activates the duodenal hormones secretin and cholecystokinin which stimulate the pancreatic secretion of bicarbonate and contraction of the gall-bladder, respectively¹⁷¹. Furthermore, gastric acidity inhibits the antral release of gastrin in a feedback mechanism. However, the main function of gastric acid is disinfection of the gastric content.

The number of bacteria present in the gastric juice correlates to the acidity of the gastric juice¹⁷³⁻¹⁷⁶. An intragastric pH below 3 to 4 is bactericidal¹⁷⁵⁻¹⁷⁸. Above pH 4, mainly *Lactobacilli* spp. and *Streptococci* spp. will proliferate, and above pH 5 a more complex flora is able to proliferate¹⁷⁸. In these situations mostly oropharyngeal bacteria, including *viridans Streptococci*, *Staphylococci* spp., *Micrococci* spp., *Neisseria* spp., *Haemophili* spp. and *Lactobacilli* spp.^{176,179,180}, faecal streptococci and *Bacteroides* spp. can be found, and sometimes Enterobacteriaceae are present^{174,176,178}.

During a food intake the gastric pH rises and bacterial growth can occur. Feeding also increases the number of bacteria in the upper small intestine¹⁷⁵. However, a fall in pH below 4.0 for more than two hours each day will prevent colonization¹⁷⁸. In subjects with a continuously reduced intragastric acidity, bacterial overgrowth can occur.

Clinical conditions associated with reduced intragastric acidity and bacterial overgrowth

In most parts of Asia, Africa, South and Central America and even in Eastern and Southern Europe, a remarkable proportion of young adults are found to be achlorhydric¹⁷⁸. This may result from protein malnutrition, virus infections or high salt intake¹⁷⁸. Also aging is associated with a decline in gastric acid output^{181,182}. Furthermore, some major clinical conditions are associated with reduced intragastric acidity and subsequent bacterial overgrowth.

Firstly, the intragastric pH is associated with the histological grade of gastritis¹⁸³: a reduced intragastric acidity with bacterial overgrowth can be found in patients with atrophic gastritis with or without pernicious anaemia. Stockbrügger et al.¹⁸⁴ found at least 10⁵ cfu/ml upper respiratory tract flora in all 22 patients with pernicious anaemia and in 14 of 22 patients also faecal-type bacteria were found. In

another group of pernicious anaemia patients, bacteria were cultured from gastric juice of 14 of 18 patients¹⁸⁵. The flora was found to be identical in gastric juice and in duodenal juice or biopsies^{184,185}. Dolby et al.¹⁸⁶ showed that bacterial overgrowth could be demonstrated in both gastric juice and gastric mucosal biopsies of patients with pernicious anaemia and found comparable concentrations of bacteria in both the gastric juice and the gastric mucosal biopsies.

Secondly, intragastric bacterial overgrowth has been demonstrated in patients after gastric surgery^{179,187,188}. For example, Keighley et al.¹⁸⁹ found bacterial counts to increase with the intragastric pH in patients after partial or total vagotomy with or without a partial gastrectomy. Carboni et al.¹⁹⁰ compared patients with a partial gastrectomy (Billroth I or Billroth II) with normal controls and found a higher pH, higher total viable counts and higher concentrations nitrate-reducing bacteria in the patients compared to the controls with the highest values after a Billroth II resection. Besides, the bacterial flora in the stomach of these patients will also be influenced by alterations in motility^{190,191} and/or the presence of blind loops¹⁷⁹.

A final important clinical cause of hypochlorhydria and achlorhydria is the use of gastric acid inhibitory drugs. Bacterial counts are negatively correlated to basal acid output after short term treatment with antimuscarinic drugs where both oropharyngeal and faecal-type bacteria were found¹⁹². Also after treatment with H₂-antagonists a rise in total intragastric and in nitrate-reducing bacteria was observed^{188,193-195}. Similar results of bacterial overgrowth are obtained after treatment with omeprazole¹⁹⁶⁻¹⁹⁸. In ambulatory patients treated with omeprazole, bacterial overgrowth of both oral and faecal-type bacteria is also found in the duodenum¹⁹⁹. Houben et al.²⁰⁰ compared the effects of 2 weeks treatment with placebo, ranitidine 300 mg, omeprazole 20 and omeprazole 40 mg and found a similar increase in bacterial growth after each form of acid-inhibition although different median 24-hour pH values are obtained. Also Muscroft et al.¹⁷⁶ could not find consistent viable bacterial counts per pH-value. Others however, found a close association between the bacterial counts and the intragastric pH^{189,195}.

Consequences of intragastric bacterial overgrowth

Bacterial overgrowth in the stomach can be associated with some short- and long-term consequences. Firstly, patients with achlorhydria and bacterial overgrowth have a higher risk for gastrointestinal infections²⁰¹. A higher risk for bacterial gastrointestinal infections has been shown after gastric surgery²⁰²⁻²⁰³, and in patients taking acid-suppressive drugs^{203, 204}. In a review, Cook²⁰⁵ summarized studies on the consequences of hypochlorhydria on gastrointestinal infections and reported increased risks for bacterial infections such as *Salmonella*, *Campylobacter*, *Shigella* and *Cholera* but also a higher predisposition for parasitic infections such as giardiasis

and strongyloidiasis. Finally a reduced intragastric acidity is linked to candidiasis^{206,207}.

A second consequence of bacterial overgrowth is the risk of a bacterial overgrowth syndrome²⁰⁸. Especially small bowel bacterial overgrowth may interfere with the metabolism of nutrients, vitamins and minerals. This may result in a bacterial overgrowth syndrome characterized by malabsorption, and complications resulting from bacterial deconjugation of bile salts²⁰⁹. Bile salts can cause histological and ultrastructural damage to the gastric and intestinal mucosa²¹⁰ and thereby cause malabsorption of nutrients, lactose intolerance and protein malabsorption¹⁷⁸. Bile salt degradation results in an impaired fat absorption and steatorrhoea. On long-term there may develop a deficiency in fat-soluble vitamins. The bacterial flora can also interfere with the absorption of vitamin B12 and folate¹⁷⁸. In addition, bacterial overgrowth following acid-inhibitory drugs can interact with the absorption of iron, calcium, vitamin B12 and phosphate²¹¹.

Sherwood et al.¹⁷³ describe a marginal fat-malabsorption in half of the patients with pernicious anaemia. In a retrospective study, Stockbrügger et al.¹⁸⁴ find a history of malabsorption and/or chronic intermittent diarrhoea in one third of patients with pernicious anaemia with intragastric and intestinal bacterial overgrowth. This occurred more frequently in patients with duodenitis. In another study no signs of a bacterial overgrowth syndrome are found¹⁸⁵.

A major consequence of intragastric bacterial overgrowth is the production of nitrite and N-nitroso compounds (NNOC's). NNOC's are formed by N-nitrosation between various secondary and tertiary amines and nitrite or other nitrosating agents²¹². The total human exposure of NNOC's consist of exogenous and endogenous components. Exogenous NNOC's mainly result from tobacco products, nitrite-cured meat, medicinal drugs and by occupational exposure²¹³. In addition, humans are also exposed to many nitrogen compounds and nitrosating agents from tobacco smoke, diet and drinking water²¹³.

Ingested nitrate is absorbed in the small intestine and excreted in the urine (65-70%) and the faeces (3-10%)²¹⁴. Nitrate is secreted in saliva, gastric juice, tears, sweat and probably in the large bowel and the vagina²¹⁵. In all sites where nitrate and bacteria are present, nitrite can be formed. About a quarter is taken up from the circulation by the salivary glands and a small percentage is converted to nitrite in the oral cavity^{214,215}. Nitrate reduction can further occur in the achlorhydric stomach but also in the large bowel, the infected urinary bladder and the infected cervix^{214,215}.

NNOC's can be formed endogenously by acid-catalyzed formation, by bacteria, and by activated macrophages during inflammation^{213,216}. The endogenous

N-nitrosation is controlled by the intragastric pH, concentrations of nitrite and nitrosatable nitrogen, and the incubation time²¹⁶. Furthermore, the concentration and bacterial species present play a role in the bacterial nitrosation²¹⁶. Acid-catalyzed nitrosation occurs at a low pH (2-3) while bacterial nitrosation occurs at a pH of 6-8²¹⁷.

With an increase of the pH in the stomach, increases of both total bacterial counts and numbers of nitrate-reducing bacteria are observed in achlorhydric patients with pernicious anaemia, after gastric surgery and after gastric inhibitory therapy^{176,180,188,190,193,195}. Nitrate-reducing bacteria found in achlorhydric patients may include *Micrococci* spp., *Staphylococcus aureus*, *Haemophili* spp., *Veillonella* spp. and *Escherichia coli*^{176,180}. In gastric juice of achlorhydric patients, also increased levels of nitrite^{184,190} and of both nitrite and NNOC's have been demonstrated^{195,196,218}. Some studies found only an increase in nitrite but not in NNOC's^{188,189}. Verdu et al.¹⁹⁷ and Houben et al.²⁰⁰ did not find any significant increases in nitrate, nitrite or NNOC's after short-term treatment with omeprazole and ranitidine. The role of the intragastric pH in the nitrosation may partially explain differences in nitrite and NNOC concentrations found in diverse studies on achlorhydric patients. Furthermore, the measurements of NNOC's can be influenced by instability of NNOC's, the time between sampling and the assay, and the relative lack of specificity in certain assays^{219,220}.

NNOC's are found to be toxic in humans²¹² and the use of various nitroso-urea derivates in treatment regimens of some human forms of cancer provide evidence that they may cause cancer themselves²²¹. Furthermore, an association with cancer is demonstrated in several animal studies²²². Further evidence for a carcinogenic role of NNOC's comes from epidemiological and clinical studies which find an association between nitrate and nitrite rich diet or drinking water and an increased risk of gastric cancer^{213,223}. A role of NNOC's in the aetiology of gastric cancer is also supported by a study of Li et al.²²⁴, where a higher NNOC synthesis measured by the NPRO test, was found for inhabitants of high versus low risk areas of gastric cancer.

Also in clinical situations associated with bacterial overgrowth and high levels of nitrite and NNOC's in the stomach, an increased risk of gastric cancer is found^{115-118,120,122,225,226}. In gastric cancer patients an increased pH and high nitrite concentrations are reported²²⁷. All these findings support the possibility of a carcinogenic role of NNOC's in the pathogenesis of gastric cancer.

Detection of the intragastric bacterial flora

Helicobacter pylori

Various tests have been described for the detection of *H.pylori* in the upper gastrointestinal tract and can be divided into invasive and non-invasive tests. Invasive tests require biopsy specimens obtained during upper gastrointestinal endoscopy and include culture, histology, biopsy urease test, and polymerase chain reaction (PCR). To avoid a sampling error, at least two biopsies should be taken of both antrum and corpus²²⁸⁻²³⁰. Non-invasive tests include serology and breath tests. These will not be influenced by the patchy distribution of the bacterial flora.

Histology

For the histological detection of *H.pylori*, non-specific staining methods are widely applied such as haematoxylin-eosin stain (HE), and the modified Giemsa stain (MG)²³¹. The Warthin-Starry silver stain (WS) is more specific²³². In the HE stain, the bacteria are only faintly visible and the sensitivity is low compared to Gram stain and WS stain²³³. The fact that this bacterium has not been noticed for several decades supports the low sensitivity of the HE stain^{233,234}. The WS stain improves the recognition of *H.pylori* because the organisms appear larger than they are^{228,233}. However, this stain is expensive and difficult to perform. The MG stain is easy to perform, inexpensive, and has a high sensitivity^{233,235,236}. Some find no difference between the MG stain and the WS stain²³¹, while others find a higher sensitivity for the MG stain^{235,237,238}. Also the acridine orange stain is very effective but fluorescent microscopy is required^{239,240}.

As an advantage of histology, the presence of gastritis can be assessed simultaneously but the quality of the biopsy can influence the histological detection of the bacteria^{230,236}. Using non-specific staining techniques, the *H.pylori* organism has to be identified by its appearance and typical location within or beneath the gastric mucus layer^{228,233}. Other coccoid, rod-like or spiral shaped bacteria can be present in the stomach which may be difficult to differentiate from *H.pylori*. Both sensitivity and specificity of non-specific histological staining methods for the detection of *H.pylori*, will depend on the experience and critical view of the observer²³⁰ and can only assess the presence of *H.pylori*-like organisms.

Culture

Culture is very specific for the detection of *H.pylori* but is not always found to be as sensitive as histology, although good results can be achieved when the specimens

are rapidly and correctly transported and processed on appropriate media²⁴¹. Saline is a convenient transport medium provided that the specimens are cultured within four hours and kept at 4°C^{236,242}. Use of fresh media with fresh blood, a moist micro-aerophilic atmosphere and incubation periods of 4-7 days are required^{233,236,242}. Heavier growth is obtained when biopsy specimens are ground compared to smearing them over the culture medium²³⁶. The presence of other bacteria in a biopsy specimens can disturb the detection of *H.pylori* and can be reduced by use of selective media²⁴³. Those bacteria mainly consist of oropharyngeal flora and Gram negative rods found in patients with impaired gastric acid secretion. Selective media with antibiotic combinations consisting of vancomycin, trimethoprim and polymyxin B or a combination of vancomycin, nalidixic acid and amphotericin B have been applied. However nalidixic acid²⁴⁴ and polymyxin B²⁴⁵ sensitivity has been described for *H.pylori*. These antibiotics can be replaced by cefsulodin. The advantage of culture is that antibiotic sensitivity can be assessed simultaneously. This can be very valuable when eradication therapy for *H.pylori* has failed²²⁹.

Biopsy urease test

A very quick invasive method is the biopsy urease test. This test is based on the high urease activity of *H.pylori*: Urea will be hydrolysed leading to the production of ammonia and carbonate both producing an increase of the pH. This increase can be visualized using a pH indicator in the medium. Most urease tests have a good specificity but a wide range of sensitivity is described²⁴¹. Sensitivity depends on the number of bacteria present in the specimens: a quantity below 10⁴ microorganisms may result in a negative urease test²⁴¹. The sensitivity will decrease when patients are treated with antibiotics or gastric acid inhibitors²⁴¹. The biopsy-urease tests will be very specific but not very sensitive when read after 1 hour. Sensitivity will increase but specificity will decrease when the test is read after 24 hours²⁴⁶. Especially after 24 hours, false positive results occur by the presence of contaminating bacteria with a lower urease activity²⁴⁶. However, others do not find false positives when the test is read after 24 hours²⁴⁷. In short, the sensitivity and specificity of this test is strongly dependent on the media used and the incubation time^{233,241,245,246}.

Polymerase Chain Reaction

In recent years, the polymerase chain reaction (PCR) is widely applied for the detection of *H.pylori* in gastric biopsy specimens. PCR can for example be based on the urease A gene^{248,249} or 16s rRNA^{250,251}. A high sensitivity and specificity was found performing PCR on several clinical isolates of *H.pylori* in comparison with

many other bacterial strains^{248,249,252}. Furthermore, several studies found PCR to be superior to culture, histology^{248,250,251}, the urea breath test²⁵³, the biopsy urease test and serological Western blotting²⁵¹. As an additional advantage, PCR can be used for the detection of *H.pylori* in gastric juice²⁴⁹, dental plaque^{37,38}, faecal samples³⁴ and on endoscopes²⁵⁴.

Urea breath test

The urea breath test is also based on the fact that the urease enzyme hydrolyses urea producing carbon dioxide which subsequently diffuses into the blood and thereafter is partly excreted by the lungs²⁵⁵. In the test ¹³C (non-radioactive) or ¹⁴C (radioactive) labelled urea is given orally and later detected in the breath. A high energy test meal has to be given to delay gastric emptying²⁵⁵. This will result in a prolonged exposure of urea to the gastric bacteria and may prevent the substrate from reaching the colon where colonic bacteria could release carbon dioxide²³⁶.

In general the urea breath test is well tolerated, without risk when using non-radioactive tracers (¹³C) and does not require experienced operators²⁵⁵. Good results are obtained with sensitivities from 90%-100% and specificities from 78%-100% in clinical situations^{255,256}. The urea breath test can only detect an active *H.pylori* infection. False negative results can occur after treatment with antibiotics, bismuth or omeprazole^{255,256}. Therefore, testing has to occur at least one month after the end of treatment²⁵⁵. As a disadvantage, the urea breath test does not measure bacterial load, and obviously no macroscopic and microscopic information of the stomach is obtained.

Serology

Chronic *H.pylori* infection elicits a local and systemic immune response²²⁸. The presence of serum antibodies against *H.pylori* are found to correlate with *H.pylori* associated gastritis^{236,257}. The accuracy of serological tests may vary with the antigens used and the population tested^{233,258}. Using ELISA assays, the definition of the cut-off value is important and a grey zone has to be determined where results are indeterminate²⁵⁸. The assay should be validated in a local reference population²⁵⁹ as the antibody titre may differ with ethnicity and age^{258,260,261}. Furthermore an ELISA assay is less accurate in patients using NSAIDs²⁶².

Patients may have a positive serology but negative histology or culture as they can have had a previous infection leaving a serological scar. This can occur after *H.pylori* eradication or on the basis of developing gastric mucosal atrophy or intestinal metaplasia which both are hostile to *H.pylori*²⁶⁰.

The antigens used in an ELISA assay are not allowed to be present in other bacteria such as *Campylobacter jejuni*, *Campylobacter fetus* or *Escherichia*

coli^{236,263} and antibodies against different *H.pylori* strains have to be applied to improve both the sensitivity and specificity of the assays. The antibody titres decrease slowly after eradication therapy for *H.pylori*^{228,236}. For checking eradication of *H.pylori*, antibodies have to be determined before and not earlier than three months after treatment to find a sensitivity and specificity in an acceptable range²⁶⁴.

Non-*Helicobacter pylori* bacterial overgrowth

Culture

Gastric and duodenal juice and biopsies can be obtained for culture of the total aerobic and anaerobic bacterial flora using both selective and non-selective agar-plates. In intestinal aspirate, bacterial overgrowth is confirmed by the presence of at least 10^5 colony forming units per ml²⁶⁵. Although culture of gastric and intestinal aspirates have been widely applied, to our knowledge, Dolby et al.¹⁸⁶ and Stockbrügger et al.¹⁸⁵ are the only ones, who cultured gastric and duodenal mucosal biopsies to assess the presence of the non-*H.pylori* bacterial growth. In both studies biopsy specimens were washed three times and were ground before inoculation.

Breath tests

A non-invasive method for the detection of bacterial overgrowth is the application of breath tests. Both ¹⁴C- and H₂-breath tests are used for the detection of intestinal bacterial overgrowth, as bacteria can deconjugate bile-salts and ferment carbohydrates.

Administration of radio-labelled ¹⁴C-glycocholate results in increased levels of ¹⁴CO₂ in the expired breath^{266,267}. However, this method takes six hours and cannot be used in patients with ileal diseases^{265,268}. Better results in the detection of small-intestinal bacterial overgrowth are obtained by using the 1 gram ¹⁴C xylose breath test²⁶⁹.

Bacteria can also ferment carbohydrates with the production of hydrogen, which is absorbed, and can partly be exhaled. After overnight fasting, either a glucose, lactose or lactulose solution is given and H₂ production can be measured. False negative results may occur when using glucose because this is rapidly absorbed. Better results can be obtained with lactulose which is poorly absorbed and can distinguish between small-bowel bacterial overgrowth and ileal diseases^{265,268}. However, acceleration of small-intestinal transit results in false positive results by colonic fermentation of substrate and false negative breath tests can occur in patients receiving antibiotics and in non-H₂-producers²⁷⁰.

The hydrogen-breath test can also be used for the detection of intragastric bacterial overgrowth¹⁸⁷. However, an unchanged H₂-breath test is found after treatment with cimetidine while an increase of the bacterial concentration was found in gastric juice²⁷¹.

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Aims of the study

A *Helicobacter pylori* (*H.pylori*) infection is associated with duodenal and gastric ulcers, gastric cancer and primary gastric lymphoma. In clinical conditions or lesions associated with a decreased gastric acidity, a non-*H.pylori* bacterial flora can colonize the stomach. In the histological sequence of *H.pylori*-associated gastritis towards atrophic gastritis, intestinal metaplasia and gastric cancer, the intragastric pH increases. In this sequence, both *H.pylori* and/or non-*H.pylori* bacteria may be present. Until now, most attention has been paid to the presence and role of *H.pylori* in the pathogenesis of these conditions. To detect *H.pylori*, usually non-specific histological staining methods or bacteriological culture are used with a relatively low sensitivity. The role of the non-*H.pylori* flora in the above-mentioned conditions is insufficiently documented. To assess the contribution of either type of bacterial flora in the pathogenesis of the clinical conditions mentioned above, it is important to use sensitive and specific detection methods to distinguish *H.pylori* and non-*H.pylori* flora.

The studies in this thesis describe the following subjects:

- * Optimisation of culture procedures for the detection of *H.pylori* (**Chapter II**).
- * Evaluation of a specific immunohistochemical stain in comparison with non-specific staining methods for the histological detection of *H.pylori* in clinical situations where both *H.pylori* and/or non-*H.pylori* can be present (**Chapter III and IV**).
- * Use of the immunohistochemical stain and the modified Giemsa stain to assess the total intragastric bacterial flora in clinical situations associated with *H.pylori*: peptic ulcer disease (**Chapter V**), primary gastric lymphoma (**Chapter VI**) and gastric cancer (**Chapter VII**).

In all situations, the presence, gastroduodenal distribution and density of both *H.pylori* and non-*H.pylori* intragastric bacterial flora were related to clinical findings.

Chapter II

Influence of oropharyngeal flora and specimen pretreatment on the recovery of *Helicobacter pylori*

D Jonkers, E Stobberingh, R Stockbrügger

Summary

The recovery of *Helicobacter pylori* was studied *in vitro* in the presence of different concentrations of oropharyngeal flora. The effect on the bacterial yield of *Helicobacter pylori* and oropharyngeal flora by washing biopsies once, twice, or thrice before culture was then studied in biopsies taken from 35 patients. A low rate of recovery of low *Helicobacter pylori* concentrations ($\leq 10^3$ cfu/ml) was related to the presence of relatively high amounts of oropharyngeal flora ($\geq 10^4$ cfu/ml). After three washings a decrease in oropharyngeal flora between 10-fold and 100-fold cfu/biopsy was observed in seven patient samples. In another three specimens a more than 100-fold reduction in cfu/biopsy was observed. For *Helicobacter pylori*, a 60-fold decrease in log cfu/biopsy was observed in only one patient. Pretreatment of the specimens by washing of the biopsies improved the recovery of small amounts of *Helicobacter pylori* in 10 of 28 patients. For optimal detection of *Helicobacter pylori* in gastric mucosal biopsies, washing of the specimens in addition to the use of selective media is recommended.

Introduction

Helicobacter pylori (*H. pylori*), a Gram negative microorganism, has been identified in cases of duodenal and gastric ulcer disease, gastric cancer and MALT-lymphoma¹⁻³. Isolation of *H. pylori* from gastric mucosal biopsies is important to confirm the role of this microorganism in the pathogenesis of these diseases and it offers the additional advantage of having an isolate available for antibiotic susceptibility testing.

Although, it could be expected that only *H. pylori* is present in the gastric biopsy specimen, use of inhibitors of gastric acid secretion, as in many patients with duodenal and gastric ulcer disease, or progression of gastritis towards atrophic gastritis results in an increase in the gastric pH⁴, and consequently in an increase in contaminating flora from the respiratory and/or gastrointestinal tract^{5,6}. The presence of other flora may influence the detection of the fastidiously growing *H. pylori*^{7,8}. To optimize the recovery of *H. pylori* from specimens containing contaminating oropharyngeal or faecal flora, use of selective media seems most useful⁹. Another approach is washing of the specimens to remove the contaminating flora^{10,11}.

In the present study we evaluated the effect of the use of both selective media and washing of specimens on the yield of *H. pylori* from gastric biopsy specimens.

Material and methods

Influence of selective media on growth of oropharyngeal flora and *Helicobacter pylori*

Samples of 0.01 ml of suspensions (10^7 cfu/ml) of 14 clinical samples of oropharyngeal flora identified as viridans streptococci, *Neisseria* spp. and *Corynebacterium* spp. (in about equal concentrations) and 10 clinical strains of *H. pylori* were used to inoculate three selective plates with the five segments method¹². The Skirrow medium¹³ contained Columbia agar (Oxoid CM331, Oxoid, UK) with 5% sheep blood and a selective supplement (Oxoid SR069) comprising vancomycin, trimethoprim and polymyxin B. CPS plates⁷ contained blood agar base no.2 (Oxoid CM271) with 7% laked horse blood (Oxoid SR048) and Dent selective supplement (Oxoid SR147). Nal medium¹⁴ contained Columbia agar with 5% sheep blood, vancomycin 10 mg/l (Eli Lilly, The Netherlands), trimethoprim 5 mg/l (La Roche, The Netherlands), and nalidixic acid 20 mg/l (Sterling-Winthrop, UK). All plates were incubated under micro-aerophilic conditions (10% CO₂, 5% O₂) at 37°C for 5 to 7 days.

Influence of oropharyngeal flora on recovery of *Helicobacter pylori*

Oropharyngeal flora from clinical specimens as described above was used to study in vitro the influence of this flora on the rate of recovery of different concentrations of clinical isolates of *H. pylori* as follows: 1 ml of different concentrations of *H. pylori* from 10^2 to 10^5 cfu/ml in physiological saline was thoroughly mixed with 1 ml of ten-fold dilutions of oropharyngeal flora from 10^1 to 10^7 cfu/ml. Aliquots of 0.01 ml of the mixture and of both bacterial suspensions separately were used to inoculate blood agar plates (Oxoid CM271, with 5% sheep blood) and Skirrow plates selective for *H. pylori* with the five-segments method. Blood agar plates for growth of oropharyngeal flora were incubated at 37°C for two days; the *H. pylori* plates were kept under micro-aerophilic conditions (10% CO₂, 5% O₂) at 37°C for 5 to 7 days. Each experiment was performed in triplicate.

Washing the biopsy specimens

In the endoscopy department, biopsy specimens were obtained from patients (n=35) who were likely to have either bacterial overgrowth due to reduced gastric acid secretion as a consequence of atrophic gastritis or gastric surgery, or *H. pylori* due to the presence of peptic ulcer disease. From each patient four biopsies of neighbouring tissue (mean weight $\pm$ SD: 12 $\pm$ 5 mg per biopsy) were taken from antrum (n=34) or corpus (n=1), using an Olympus GIFQ gastroscope (Zoeterwoude, The Netherlands). The endoscope and the biopsy forceps were disinfected with 2% glutaraldehyde before each examination. In each patient the same biopsy forceps was used to take the four specimens. Each specimen was collected separately in a tube with 2 ml sterile saline. The washing procedure was as follows. Each tube was shaken 20 seconds with a vortex. Using sterile forceps the specimen was then transferred either to the agar plates or to a second tube with 2 ml sterile saline, and the whole procedure was repeated once or twice (corresponding to two or three washings). The five segments method was used to inoculate blood agar plates for (facultative) aerobic bacterial growth and *H. pylori* plates as described above.

Bacterial growth was assessed quantitatively by determining the number of colony-forming units (cfu). A not fully grown first segment, corresponded to 10^1 to 10^2 cfu/ml, a fully overgrown first segment to 10^3 cfu/ml, a fully overgrown second segment to 10^4 cfu/ml and so on, overgrowth in all five segments corresponded to 10^7 cfu/ml.

Identification of bacteria

For the identification of the microorganisms standard microbiological methods were used¹⁵. Oropharyngeal flora included *Streptococci* spp., *Neisseria* spp., and *Corynebacterium* spp.; faecal flora included Enterobacteriaceae and *Enterococcus faecalis*. *H.pylori* was identified initially as small translucent colonies, 1 mm in diameter, and was further identified by Gram stain and positive urease, catalase, and oxidase reactions.

Results

Influence of selective media on growth of oropharyngeal flora and *Helicobacter pylori*

In a preliminary study we studied the growth of 14 clinical samples of oropharyngeal flora and 10 clinical strains of *H.pylori* on the three types of media. Contaminating flora was almost completely inhibited on the Nal medium except in two samples. On the CPS and the Skirrow media contaminating flora was observed in 12 and 8 samples respectively, counts being higher on the CPS medium. Growth of *H.pylori* was similar on both the CPS and Skirrow media, but on the Nal medium growth of *H.pylori* was inhibited and the colonies were often smaller.

The higher number of samples with contaminant growth on the CPS medium is in accordance with the antibacterial activity of the antibiotics used. Polymyxin B has good antibacterial activity against both *Pseudomonas* spp. and Enterobacteriaceae, and cefsulodin has especially good activity against *Pseudomonas* spp. but lower activity against Enterobacteriaceae. As the aim of our study was an optimal isolation of *H.pylori* with minimal contaminant flora we decided to test the effects of washing using only the Skirrow medium.

Recovery of *Helicobacter pylori* in the presence of oropharyngeal flora

When *H.pylori* concentrations were $\geq 10^4$ cfu/ml, the recovery of this organism was not influenced by the presence of oropharyngeal flora in concentrations ranging from 10^1 to 10^7 cfu/ml. However, when *H.pylori* concentrations were $\leq 10^3$ cfu/ml, the recovery was markedly reduced in the presence oropharyngeal flora in concentrations of 10^3 cfu/ml or higher (Figure II.1).

Figure II.1: Recovery of *Helicobacter pylori* (A, original concentration 10³ cfu/ml; B, original concentration 10⁵ cfu/ml) added to different concentrations of oropharyngeal flora

Pretreatment of the specimens by washing

The bacteria isolated from the 35 biopsies comprised oropharyngeal flora in 28, *H.pylori* in 17, and faecal flora in 7 biopsies. Both oropharyngeal flora and *H.pylori* were present in 15 biopsies (Table II.1). In the *H.pylori* positive patients, *H.pylori* counts were low ($\leq 10^3$ cfu/biopsy) in 8 of 17 patients.

Table II.1: Bacterial flora in gastric biopsies from 35 patients before washing

	No. of	Counts (cfu/biopsy)	
Bacterial flora	biopsies	median	concentration
Flora or <i>H.pylori</i>			
Oropharyngeal flora	28	8.2x10 ²	(2 - 3.0x10 ⁶)
<i>Helicobacter pylori</i>	17	5.0x10 ³	(8 - 1.0x10 ⁷)
Faecal flora	7	1.9x10 ⁴	(2 - 2.5x10 ⁸)
Flora + <i>H.pylori</i>			
Oropharyngeal flora	15	1.3x10 ³	(2 - 3.0x10 ⁶)
<i>Helicobacter pylori</i>		5.0x10 ³	(8 - 1.0x10 ⁶)

Table II.2: Number of patients with a 10-fold or 100-fold decrease in cfu/biopsy after one, two or three washings compared to no washing for oropharyngeal flora (OF) (n=28) and *H.pylori* (n=17)

Decrease	One washing		Two washings		Three washings	
	OF	<i>H.pylori</i>	OF	<i>H.pylori</i>	OF	<i>H.pylori</i>
No decrease	14	14	11	11	8	11
≤ 10 -fold	10	3	9	6	10	5
> 10 to ≤ 100 fold	4	-	5	-	7	1
> 100 -fold	-	-	3	-	3	-

The effect of washing the biopsies before inoculation on the yield of oropharyngeal flora and *H. pylori* is shown in Table II.2. After three washings an at least 10-fold decrease in the counts of oropharyngeal flora was observed in the biopsies of 7 of 28 patients. In another three patients an at least 100-fold decrease was found. Oropharyngeal flora was still present in relatively low counts after three washings in 26 of 28 patients. In the case of *H. pylori*, a 60-fold reduction was observed in the specimen of only one patient after three washings. Pretreatment of the specimens by washing of the biopsies improved the recovery of small amounts of *H. pylori* in at least 10 of 28 patients.

Discussion

The main feature of the present study was to evaluate the use of both selective media and washing of specimens before inoculation on the rate of recovery of *H. pylori* in biopsy specimens. In 10 of 28 patients the recovery of small numbers of *H. pylori* was improved by washing the specimens prior to inoculation.

In the past decade several different selective media have been reported to optimize the yield of *H. pylori* and to reduce the influence of other contaminating flora. The Skirrow medium used in this study contains vancomycin, trimethoprim and polymyxin B, and is suitable for isolation of the majority of *H. pylori* strains^{7,13}. The antibiotics in this medium are considered to be inhibitory for oropharyngeal and faecal flora as vancomycin inhibits all Gram-positive microorganisms, and trimethoprim and polymyxin B inhibits Gram-negative rods i.e. *Haemophilus* spp., Enterobacteriaceae and *Pseudomonas aeruginosa*. As some polymyxin B susceptible *H. pylori* strains have been described¹⁶, the Skirrow selective medium might be inhibitory for these microorganisms. An alternative supplement described by Goodwin et al.¹⁴ contains nalidixic acid and amphotericin B instead of polymyxin B. Nalidixic acid reduces the growth of Enterobacteriaceae and *Pseudomonas* spp. Amphotericin B was added to inhibit growth of *Candida* spp. which are often present in the oropharyngeal flora. As nalidixic acid susceptible *H. pylori* strains have been reported¹⁷, this supplement might also be inhibitory for these strains. Goodwin et al.¹⁴ reported good results in terms of isolation of *H. pylori* and inhibition of contaminants using this medium in combination with 1% Isovitalex and 7% horse blood. Dent and McNulty¹⁷ evaluated a CPS medium which contains cefsulodin and amphotericin B. Comparing the CPS and the Skirrow media, they concluded that the CPS medium did not inhibit *H. pylori* and that the colonies were often larger and more easily recognized on it. They reported growth of contaminating flora on CPS medium in 11 biopsies compared to growth

on Skirrow medium in 5 biopsies. We observed optimal isolation of *H.pylori* with the lowest rate of contaminating flora using the Skirrow selective medium compared to CPS and Nal media. Therefore, we used the Skirrow medium to study the effect of washing.

The endoscopic procedures applied in our endoscopy unit to obtain biopsies are optimal and not expected to influence culture results¹⁸. All biopsies were kept in sterile saline at 4°C and cultured within four hours. These conditions were in accordance with those described by others^{19,20}.

This study shows that the detection of *H.pylori* by culture can be disturbed by the presence of high amounts of oropharyngeal flora ($\geq 10^4$ cfu/ml). Although this findings was obtained by in vitro experiments, clinical observations support this finding. Stolte and Bethke⁸ found a reduction of *H.pylori* in the antrum after profound acid inhibition and suggested that bacterial competition might be responsible for this.

On the basis of these findings we recommend that specimens are washed before inoculation in addition to the use of Skirrow medium for optimal isolation of *H.pylori* from gastric biopsy specimens. Further studies with large number of patient samples will be needed to demonstrate significantly the positive effect of washing the specimens on the recovery of *H.pylori*.

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Chapter III

Evaluation of immunohistochemistry for the detection of *Helicobacter pylori* in gastric mucosal biopsies

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Summary

A reliable diagnosis of *Helicobacter pylori* (*H.pylori*) is important in clinical practice and research. To evaluate sensitivity, specificity and inter-observer variation of three histological staining methods for the diagnosis of intragastric *H.pylori* bacterial flora, four observers assessed the presence of *H.pylori* in a test set of pairs of gastric biopsies taken from 40 patients during GI-endoscopy. Histological slides of one biopsy of each patient (formalin fixed and paraffin embedded) were stained with a modified Giemsa (MG), the Warthin-Starry (WS), and an immunohistochemical method (IMM) using purified polyclonal *H.pylori* antiserum (DAKO B471). As a standard bacterial culture was performed on the adjacent biopsy, using the four quadrants method. Special attention was paid to the presence of non-*H.pylori* bacterial flora.

Twenty out of 40 specimens were *H.pylori* positive by culture. Using culture as a standard sensitivity for *H.pylori* was 90.0 ± 10.0 % with MG, 70.0 ± 14.1 % with WS, and 83.8 ± 11.1 % with IMM stain. Specificity was 53.8 ± 19.3 , 82.5 ± 9.6 and 90.0 ± 0.0 , respectively. Of 37, 14 and 8 false positive scores by all observers for MG, WS and IMM, respectively, non-*H.pylori* flora was cultured from 17, 6, and 4 of the corresponding adjacent biopsies. Non-*H.pylori* flora may account for overdiagnosis of *H.pylori* in gastric biopsies. Kappa values for the variance between the four observers were 0.28 for MG, 0.57 for WS, and 0.83 for IMM.

Immunohistochemical staining for *H.pylori* is highly specific and has a low inter-observer variation. We advise that in situations where gastric histology is the main diagnostic tool IMM should be used for the detection of *H.pylori*.

Introduction

In the recent decade *Helicobacter pylori* (*H. pylori*) has been associated with chronic atrophic gastritis of type B, duodenal and gastric ulcers, and also with the pathogenesis of gastric cancer and MALT lymphoma¹⁻⁵. Extensive research in this field is ongoing.

However, it is known that also other bacteria (Gram-negative and Gram-positive) can be found in the gastric lumen, more permanently in states of reduced gastric acid secretion such as achlorhydric atrophic gastritis type A^{6,7}, after gastric surgery⁸⁻¹⁰, and (recently of growing importance) after profound medical acid inhibition¹¹⁻¹³. However, also in normal subjects transient presence of bacteria in the stomach can be expected after every meal before the gastric acid has effected its bactericidal action¹⁴.

Although it is known that non-*Helicobacter pylori* (non-*H.pylori*) bacterial flora can exist concurrently with *H.pylori*, little attention has been paid to the confounding effect of such flora in the histological detection of *H.pylori*.

It was the aim of this study, to compare sensitivity, specificity and inter-observer variation of the modified Giemsa, Warthin-Starry silver stain, and immunohistochemistry in single biopsies for the detection of *H.pylori*, in the presence or absence of non-*H.pylori* flora irrespective of the clinical context. For the purpose of this study culture of gastric mucosal biopsies was used as a standard.

Material and methods

Specimens

Gastric biopsies were obtained from patients (n=40) attending the Department of Gastroenterology for open access endoscopy. Using an Olympus GIFQ gastroscope (Zoeterwoude, The Netherlands), two neighbouring biopsies were obtained, at most 1 cm apart, from antrum (n=37) or body (n=3). The first biopsy was used for bacteriological culture and the second for histological staining, i.e. modified Giemsa stain (MG), Warthin Starry silver stain (WS), and immunohistochemical stain (IMM).

Culture

The biopsies for culture were transported in sterile saline (0.9%), kept at 4°C, and cultured within 4 hours as described previously¹⁵. In short, after washing each

specimen twice, a blood agar plate (Oxoid CM271 with 5% sheep blood) for total (facultative) aerobic flora and a selective *Campylobacter* plate (Columbia agar base (Oxoid CM331) supplemented with 5% sheep blood and Skirrow selective supplement (Oxoid SR069E) for the detection of *H. pylori* were inoculated by four quadrants method¹⁶. Blood agar plates were incubated aerobically for 2 days and CAM plates micro-aerophilically for 4-7 days. Standard microbiological methods were used for the quantification and identification of the bacteria¹⁷. *H. pylori* was identified by the appearance of small translucent colonies on the agar-plate and confirmed by Gram stain as well as positive test results for urease, catalase and oxidase activity.

Histological staining

Biopsies for histological examination were formalin fixed and paraffin embedded. Serial sections (4 μ m) were cut onto three glass slides for MG, WS, and IMM, respectively. The MG and WS staining techniques were performed as described by Gray et al.¹⁸ and Warthin and Starry¹⁹, respectively. For the IMM a purified polyclonal *H. pylori* antiserum was used (DAKO B471; ITK diagnostics BV, Uithoorn, The Netherlands) in a 1:500 dilution. Staining was performed without pepsin treatment and with biotin-conjugated swine-anti-rabbit-antibodies (1:1000) (DAKO E431) as second layer, followed by streptABC-HRP (1: 1000) (DAKO K377). Sections were stained with diaminobenzidine (DAB) with 10% imidazole to intensify the reaction and they were counterstained with haematoxylin. To test cross reactivity of the polyclonal *H. pylori* antiserum with other microorganisms, suspensions of *H. pylori*, *Campylobacter jejuni*, *Campylobacter coli*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Streptococcus viridans* and *Staphylococcus aureus* as well as tissue sections of the descending colon, the sigmoid colon and the duodenum were stained immunohistochemically. Positive and negative control slides were always included. The negative controls included both a *H. pylori* negative specimen and a *H. pylori* positive specimen treated as described above without the primary antiserum.

Scoring and (statistical) analysis

All histological slides were scored blindly for the presence of *H. pylori* by four observers, i.e. two microbiologists (observers 1 and 2) and two pathologists (observer 3 and 4). All observers had a training in the assessment of *H. pylori* on histological slides. *H. pylori* was characterised by the appearance of a S-shaped rod and the localisation in the gastric mucus layer on the surface epithelium as well as within the mucus layer. Two sections of each biopsy were examined completely at 400x magnification. In case of doubt a 1000x (oil immersion) magnification was

used.

With culture as standard method sensitivity, specificity, positive (PPV) and negative predictive value (NPV) of each staining method, presented as a mean of the four observers, were analyzed statistically with an ANOVA-test and Student t-test.

Concordance between observers in scoring *H.pylori* positive or negative for each staining method is presented according to the presence of *H.pylori* and non-*H.pylori* flora by culture. Inter-observer variation for the total score of each stain is given by kappa values calculated for four observers as described by Schouten et al.²⁰.

Results

Of 40 patients biopsy specimens were obtained. Endoscopic diagnosis, histology of the biopsy, treatment before endoscopy, pH of gastric juice, and culture results are presented in Table III.1.

Crossreactivity

Suspensions of 40 different *H.pylori* strains stained positive by IMM (brown) within one minute of incubation with DAB. In contrast, only a very light brown staining was visible after two to four minutes with *Campylobacter jejuni* and *Campylobacter coli*. No cross reactivity was seen with the other bacterial species tested. Sections of descending colon, sigmoid, and duodenum (obtained from three, two and three patients, respectively) did not show a positive staining with the IMM.

Culture results

By culture, 20 out of 40 biopsy specimens were found to be *H.pylori* positive and 20 were *H.pylori* negative. Non-*H.pylori* flora was present by culture in a total of 22 specimens (11 *H.pylori* positive and 11 *H.pylori* negative specimens) always including oropharyngeal flora such as *Streptococci viridans*, *Neisseria* spp. and *Corynebacterium* spp., and twice *Escherichia coli* in addition.

The median gastric pH in patients with specimens positive for non-*H.pylori* alone was 7.0 compared to 2.5 when no bacterial flora was cultured. When *H.pylori* was cultured, alone or in combination with non-*H.pylori*, the median pH values were 1.8 and 5.8, respectively.

Table III.1: Clinical data and culture results (log cfu/biopsy) of patients included (n=40)

pat nr	endoscopic diagnosis	histology antrum/corpus	treatment	pH g.juice	culture Hp	culture nonHp
1	normal	cai	omeprazole	7.0	0.30	5.48
4		ci,im	ranitidine	6.0	2.00	1.65
12		nor	cimetidine	1.0	-	-
14		nor	-	1.5	-	0.70
15		nor	-	2.0	-	1.00
17		cai	ranitidine	7.0	5.00	3.49
18		nor	-	7.0	-	1.00
19		nor	omeprazole	5.0	-	-
27		cai,atr	-	7.0	-	-
40		cai	-	-	-	4.00
2	gastritis	cai,aty	ranitidine	1.5	3.00	3.04*
3		nor	-	1.5	-	1.88
5		ci	ASA	7.0	-	2.23
7		cai	omeprazole	5.0	-	0.85
8		cai,im	omeprazole	-	-	2.20
11		cai,reg	ranitidine	5.5	3.00	0.78
16		cai,atr	ASA	5.5	5.00	1.62
22		cai,reg	omeprazole	4.5	4.00	1.48
26		nor	-	2.0	-	-
33		cai	-	2.0	4.00	-
35		cai	-	2.0	-	-
36		ci	-	-	5.00	-
37		cai	-	-	2.00	2.00
44		ci,im	omeprazole	7.0	-	3.18
45		cai	omeprazole/bismuth	3.0	6.00	1.32
13	duodenal ulcer	cai	ranitidine	1.5	7.00	-
28		cai	-	-	3.00	-
31		cai,reg	-	1.5	***	-
9	gastric ulcer	cai,reg	omeprazole	7.0	-	1.30**
20		cai	ranitidine	6.0	3.70	2.08
30		cai,im	cimetidine	-	4.00	-
38		cai	calciumacetylsalicylate	1.0	5.00	-
39		cai	NSAID	1.5	3.00	-
43		cai,im	-	5.5	4.00	-
23	bulbitis	ci	omeprazole	7.0	-	3.32
10	oesophagitis	nor	-	3.0	-	-
21		ci	famotidine	6.5	5.00	2.34
29	carcinoma	cai,im	-	-	-	-
32		cai,im	antacid	5.5	-	-
34		cai	antacid	5.5	2.06	-

* Ecoli 0.48; ** Ecoli 1.00; *** Gram stain was positive.

Histology: nor=normal; c(a)i=chronic (active) inflammation; im=intestinal metaplasia; atr=atrophy; reg=regenerative changes; aty=atypia.

ASA = acetyl salicylic acid

Histological staining

True and false positive findings for the detection of *H. pylori* by MG, WS, and IMM for each of the four observers, based on culture results, are presented in Table III.2. The highest number of false positive scores for all observers, either pathologists or microbiologists, was obtained with the MG. Distinctly less true and false positive results were found with the WS whereas the IMM showed a high score of true positives combined with a low proportion of false negatives. Table III.3 shows mean percentages of sensitivity, specificity, PPV, and NPV of each staining method averaged from all four observers. A significantly lower specificity was found with MG (53.8%) compared to WS (82.5%) and IMM (90.0%). The sensitivity was lowest for WS (70.0%) compared to MG (90.0%) and IMM (83.8%).

Table III.2: True and (false) *H. pylori* positive slides of modified Giemsa (MG), Warthin-Starry (WS) and immunohistochemistry (IMM) for each observer*

Observer	MG	WS	IMM
1	19 (5)	16 (2)	19 (2)
2	19 (13)	16 (6)	16 (2)
3	19 (12)	14 (4)	18 (2)
4	15 (7)	10 (2)	14 (2)

* Based on culture results: *H. pylori* positive (n=20), *H. pylori* negative (n=20)

Table III.3: Sensitivity, specificity, positive and negative predictive value (%) of each staining method for the detection of *H. pylori* (mean $\pm$ SD of four observers) in 40 single gastric biopsies with culture as standard

	MG	WS	IMM
sens	90.0 $\pm$ 10.0	70.0 $\pm$ 14.1	83.8 $\pm$ 11.1
spec ^{1,2}	53.8 $\pm$ 19.3	82.5 $\pm$ 9.6	90.0 $\pm$ 0.0
ppv ^{1,2}	67.0 $\pm$ 8.9	80.7 $\pm$ 7.0	89.2 $\pm$ 1.3
npv	85.6 $\pm$ 9.3	74.2 $\pm$ 7.6	85.4 $\pm$ 8.7

1) Anova: $p < 0.01$, 2) Student t-test: spec MG-WS $p = 0.05$; spec MG-IMM $p = 0.03$; ppv MG-IMM $p = 0.02$

Of the 37 false positive scores in the MG by the four observers, oropharyngeal flora was cultured in 17 occasions including once *Escherichia coli*. The remaining 20 occasions were culture negative both for *H.pylori* and non-*H.pylori* bacterial flora. Of the total 14 and 8 false positive slides in the WS and IMM, respectively, non-*H.pylori* flora was cultured in 6 and 4 occasions.

The concordance between the four observers for MG, WS and IMM in the presence or absence of *H.pylori* and non-*H.pylori* by culture are shown in Table III.4. In total, concordance between the four observers in scoring *H.pylori* positive or negative was 50.0%, 62.5% and 85.0% for MG, WS and IMM, respectively. Especially in the absence of *H.pylori* by culture, concordance between observers was much lower in MG compared to WS and IMM. When *H.pylori* was present by culture, concordance between observers was lowest for WS compared to MG and IMM.

Presented by the kappa values, the overall agreement for MG, WS and IMM in all 40 biopsies by the four observers was 0.28, 0.57 and 0.83, respectively. Thus, the variation between observers was highest for MG and lowest for IMM.

Table III.4: Concordance between four observers in scoring *H.pylori* positive or negative per staining method in the presence or absence of *H.pylori* and non-*H.pylori* flora by culture

Culture results	modified Giemsa		Warthin Starry		Immunostaining	
Hp +/nonHp-	7/9	(77.8%)	4/9	(44.4%)	6/9	(66.7%)
Hp-/nonHp-	5/11	(45.5%)	9/11	(81.8%)	11/11	(100%)
Hp +/nonHp+	7/11	(63.6%)	6/11	(54.5%)	8/11	(72.7%)
Hp-/nonHp+	1/9	(11.1%)	6/9	(66.7%)	9/9	(100%)
Total	20/40	(50.0%)	25/40	(62.5%)	34/40	(85.0%)

Discussion

The present study showed that in single gastric biopsies, the IMM is the most sensitive and specific staining method for the histological detection of *H.pylori* compared to the MG and WS stains. In addition, using IMM, the lowest inter-observer variation was found concerning the presence of *H.pylori*.

To investigate the reliability of the histological diagnosis of *H.pylori* we chose a random test set of pairs of adjacent biopsies from 40 patients with different

endoscopic diagnoses, amongst them disorders in which *H.pylori* and/or non-*H.pylori* flora could be expected. They included cases with high gastric pH with or without medical gastric acid inhibition. Culture of the biopsies confirmed in many cases that *H.pylori* and non-*H.pylori* flora were present simultaneously. Tissue concentrations of non-*H.pylori* flora were on average somewhat lower than those of *H.pylori*, but could exceed the latter in single cases. In view of the similar preparation of the biopsies before culture, such as washing twice, one may conclude that the non-*H.pylori* flora identified not only is due to transient contamination, but can supposed to be resident in the gastric mucosa.

A gold standard for the detection of *H.pylori* is not available, neither with direct nor with indirect test methods²¹. Bacteriological culture of *H.pylori* is usually considered to be very specific but not highly sensitive²²⁻²⁴. In view of this, we have paid special attention to pretreatment of the specimens and culture media¹⁵. However, using culture as a standard we were aware of the possibility of some false negative results. These could be due to low numbers of *H.pylori* present in the specimen or to non-viable organisms²⁵. Furthermore, sampling error can occur when biopsies are taken with some distance from each other. Two patients (nr 31 and 40) were scored positive for *H.pylori* with all staining methods, but were negative on culture. These culture results may be falsely negative, especially in patient nr 31 where *H.pylori* was also seen on Gram stain. However, exclusion of these cases (5% of all) from the calculations would not essentially have changed the conclusions.

Comparing our culture techniques with those reported by Loffeld et al.²⁶ from the same laboratory, we have to comment that in the latter study neither the culture methods for *H.pylori* were optimal, nor a special effort was made to detect non-*H.pylori* bacterial flora. However, also in that study IMM was highly specific and more sensitive than MG. Furthermore, using positivity of any two of the four detection methods (culture, IMM, MG, or WS) as a standard to overcome possible sensitivity problems by culture, will not essentially change the conclusions concerning sensitivity and specificity of IMM, MG and WS.

The main objective of the present study was to compare three histological detection methods for *H.pylori* in one specimen and not to assess the *H.pylori* status of the patients. Therefore, no pathophysiological parameters, extra biopsies, or additional (non-invasive) tests such as the urea-breath test, were used as they were suggested by Cutler et al.²¹.

The MG stain is most frequently used for the histological detection of *H.pylori* as it is easy to perform²⁷⁻²⁹. In the present study the MG falsely suggested the presence of *H.pylori* not only in biopsies exclusively containing non-*H.pylori* flora, but also in some others being culture-negative for both *H.pylori* and non-

H.pylori. This might indicate that with the MG stain also non-bacterial staining artefacts, such as dye precipitation^{27,28}, can confound the *H.pylori* diagnosis.

The WS silver stain is a cumbersome and expensive technique, and background precipitation of silver may be difficult to differentiate from bacteria^{28,29}. Compared with the MG a better^{27,28}, similar³⁰, or worse^{18,24,29} identification of *H.pylori* has been reported in the literature. In the present study the WS obtained a middle position between MG and IMM with respect to the specificity. The sensitivity of WS was low compared to MG and IMM. That may lead to under-diagnosis of *H.pylori*.

The IMM method applied in this study was based on a commercially available antibody, the specificity of which had been documented by others, who had not found any cross reactivities²⁵. We found a slight crossreactivity with *Campylobacter* spp., a bacterium which is however unlikely to occur in the gastric lumen³¹. With the IMM both a high sensitivity and a high specificity were obtained, resulting in a negative predictive value for the presence of *H.pylori* comparable to that of the MG and the highest positive predictive value.

What are the clinical and scientific consequences of our findings? Most clinicians will be able to make a diagnosis of current *H.pylori* gastric infection with the help of multiple biopsies, culture, CLO test, serology and application of breath tests. Hence, histology is the method most frequently used. Optimal sensitivity and specificity should be granted. In this study two confounding factors of histological *H.pylori* diagnosis were investigated: the presence of non-*H.pylori* flora and the inter-observer variation. Both factors seem to be connected. Presence of non-*H.pylori* flora occurs in multiple occasions and false interpretations can be overcome by IMM. Non-*H.pylori* flora may particularly contribute to the inter-observer variation known for MG³². We could show that IMM had the lowest inter-observer variation. This has recently been confirmed by Few et al.³³.

In conclusion, we advise that in situations, where histology is the main diagnostic tool for the detection of *H.pylori*, IMM should be routinely used.

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Chapter IV

Ongoing gastric acid inhibition is a confounding factor in *Helicobacter pylori* diagnosis

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Summary

Eradication of *Helicobacter pylori* (*H.pylori*) by antibiotics in combination with gastric acid inhibition can result in overgrowth of non-*H.pylori* bacterial flora. This may confound the histological detection of *H.pylori* at eradication control if non-specific staining methods are used.

In 18 patients treated with amoxycillin (2 weeks) and omeprazole (6 weeks), endoscopically obtained gastric juice was cultured and two biopsies of corpus, antrum and duodenum were taken before and after eradication therapy (with gastric acid inhibition still going on) for culture and for histology to assess the intragastric bacterial flora. By histology, modified Giemsa (MG) and a *H.pylori* specific immunohistochemical stain (IMM) were evaluated.

Median pH of gastric juice was 1.5 (n=18) before and 7 (n=17) after eradication therapy, when patients were still on omeprazole. After therapy, culture showed a significant decrease ($p < 0.05$) in mean amount of *H.pylori* in corpus, antral and duodenal biopsies and a significant increase of non-*H.pylori* flora ($p < 0.05$) in gastric juice, corpus, antral and duodenal mucosa. With culture as a standard, 16 and 4 biopsy specimens were scored falsely positive for *H.pylori* by MG and IMM, respectively, and *H.pylori* was not detected in 23 and 13 biopsy specimens when culture was *H.pylori* positive.

Because of the possible presence of non-*H.pylori* flora after eradication therapy the use of IMM is recommended in this situation for the histological detection of *H.pylori*, especially in those patients with ongoing gastric acid inhibitory therapy.

Introduction

H. pylori has been associated with duodenal ulcers, gastric ulcers, gastritis, and also gastric cancer and mucosa-associated lymphoid tissue (MALT) lymphoma¹⁻³. Eradication therapy for *H. pylori* often consists of profound gastric acid inhibition in combination with antibiotics⁴⁻⁶. Use of gastric acid inhibitors like H₂-receptor antagonists and proton pump inhibitors can result in an increased intragastric pH with the possible consequence of bacterial overgrowth in the stomach⁷⁻⁹.

As the presence of other bacteria than *H. pylori* may confound the accurate histological detection of *H. pylori*, we assessed the presence of *H. pylori* and other bacterial flora before and after eradication therapy, when patients were still treated with strong gastric acid inhibition. In this situation, we compared the modified Giemsa stain (MG) with a specific immunohistochemical stain (IMM) for the detection of *H. pylori*.

Material and methods

Patients and treatment

Patients with a duodenal ulcer, gastric ulcer or erosive bulbitis diagnosed during endoscopy (between March 1994 and March 1995), who were planned to receive eradication therapy for *H. pylori*, were included (n=18). Eradication therapy consisted of amoxycillin 1 gr b.i.d. for 2 weeks and omeprazole 20 mg b.i.d for 6 weeks. Patients were excluded if they used gastric acid inhibitors before the first endoscopy, were not able to return for a second endoscopy at six weeks or received other treatment regimens.

Specimens

Before and at the end of therapy (after 6 weeks when patients were still on omeprazole 20 mg twice daily), gastric juice and two biopsies each of corpus, antrum and duodenum were obtained during endoscopy. One biopsy was collected in sterile saline and kept at 4°C until bacteriological culturing within 4 hours. The second biopsy was fixed in formalin, embedded in paraffin, and slides were cut for modified Giemsa stain (MG) and Immunohistochemistry (IMM). Gastric juice was used for bacteriological culture. The pH of the juice was measured using pH paper strips with grading steps of 0.5 from pH 0 to pH 14 (Schleicher & Schüll GmbH; Dassel, Germany).

Culture

Gastric juice was cultured quantitatively using the five segments method¹⁰ inoculating 1, 10 and 100 μ l on each of the following agar plates: blood, CLED (Cysteine lactose electrolytes deficient agar), *Campylobacter* and chocolate agar plates. Blood agar plates (Oxoid CM271, Basingstoke, UK, with 5% sheep blood) for total (facultative) aerobic bacterial growth and CLED (Oxoid CM423) for enhanced detection of Enterobacteriaceae were incubated at 37°C during two days. *Campylobacter* agar plates (Columbia agar base (Oxoid CM331) supplemented with 5% sheep blood and Skirrow selective supplement (Oxoid SR069E)) and chocolate agar plates (brain heart infusion, Difco 0037-05-2, Detroit, USA; with 5% sheep blood and 2% yeast extract) both for the detection of *H. pylori*, were kept under microaerophilic conditions (CO₂ 10%, O₂ 5%) at 37°C for 7 days.

All biopsy specimens were washed twice in sterile saline to reduce contaminating flora, weighed carefully, and ground in 0.4 ml sterile saline with a tissue grinder (no. 43950, ICN Flow Biomedicals NVSA; Asse-Relegem, Belgium)¹¹. After homogenization 0.1 ml was inoculated on agar plates using the five segments method.

Bacterial growth was assessed quantitatively as log colony forming units (cfu) per millilitre gastric juice or per gram tissue. In a not full-grown first segment numbers of colonies were counted (1 log to 2 log). A full grown first segment corresponded to 3 log cfu, growth in the second segment to 4 log cfu, up to growth in all five segments which corresponded to 7 log cfu. For the identification of the microorganisms standard microbiological methods were used¹².

Histology

The modified Giemsa stain was performed as described by Gray et al.¹³. For immunohistochemistry a purified polyclonal antiserum against *H. pylori* was obtained from ITK diagnostics BV (DAKO B471, ITK diagnostics BV, Uithoorn, The Netherlands). The antiserum was raised in rabbits after immunization with heat-treated cells of one *H. pylori* strain¹⁴.

Staining was performed using the polyclonal antiserum in a 1:500 dilution and biotin-conjugated swine-anti-rabbit-antibodies (1:1000) (DAKO E431) as second layer, followed by streptABC-HRP (1:1000) (DAKO K377). Sections were stained with diaminobenzidine (DAB) with 10% imidazol to intensify the reaction and were counterstained with haematoxylin. Slides were assessed blindly for the presence of *H. pylori* by the S-shaped appearance and the localisation in the gastric mucus layer at 400 magnification. If necessary a 1000x oil immersion magnification was used.

Analysis of results

Amounts of *H.pylori* and non-*H.pylori* bacterial flora detected by culture were compared statistically by the non-parametric paired Wilcoxon test. For the purpose of this study bacterial culture was used as standard to compare results by MG and IMM for the detection of *H.pylori*.

Results

Patients

Eighteen patients (12 men and 6 women; mean age 51.8 ± 11.6 SD) with a duodenal ulcer (n=13), a gastric ulcer (n=3), or erosive bulbitis (n=2) were allotted to eradication therapy for *H.pylori*. Before treatment all patients were *H.pylori* positive by either MG or by culture in antrum or corpus. Median pH of gastric juice was 1.5 (range 1.0-2.5) (n=18). After eradication therapy, when patients were still on omeprazole 20 mg twice daily, with the last dose taken about 12 hours before control endoscopy, the median pH was 7.0 (range 1.0-7.0) (n=17).

Culture results

Mean log cfu/ml gastric juice and mean log cfu/gr biopsy of all 18 patients before and after therapy for *H.pylori* and for non-*H.pylori* bacterial flora are given in Figure IV.1. *H.pylori* and non-*H.pylori* flora were detected by culture in 15 and 7 patients before therapy, and in 9 and 15 patients after therapy, respectively.

Before therapy, *H.pylori* was found in the gastric juice of only one patient; in the biopsy specimens, relatively high mean amounts of *H.pylori* were found in body (4.8 log), antrum (5.3 log) and duodenum (3.5 log). After therapy, a significant decrease in mean amounts of *H.pylori* was found in corpus (2.7 log) ($p=0.02$), antral (1.8 log) ($p=0.003$) and duodenal biopsies (0.3 log) ($p=0.001$). Before therapy, the highest amounts of *H.pylori* were found in the antrum, whereas after therapy, the highest amounts were present in the corpus biopsies.

Before therapy only small amounts of non-*H.pylori* flora were present in corpus (0.5 log), antrum (1.2 log) and duodenum (0.3 log). No growth was found in the gastric juice. After therapy, mean amounts of non-*H.pylori* flora increased significantly in gastric juice (4.5 log) ($p=0.003$) as well as in corpus (3.0 log) ($p=0.004$), antral (3.1 log) ($p=0.01$) and duodenal biopsies (3.4 log) ($p=0.001$).

Bacterial flora other than *H.pylori* consisted of oropharyngeal flora such as *Streptococci viridans*, *Neisseria* spp. and *Corynebacterium* spp.; in addition, in

three patients faecal like flora, i.e. Enterobacteriaceae, was found after therapy.

Figure IV.1: Presence of *Helicobacter pylori* (A) and non-*Helicobacter pylori* bacterial flora (B) by culture (mean $\pm$ SD log cfu per ml gastric juice or per gr biopsy) before and after eradication therapy (n=18). * Wilcoxon: $p < 0.05$

Assessment of *Helicobacter pylori* by culture, MG and IMM

For each patient the presence of *H.pylori* by culture, MG, IMM and Gram stain before and after therapy are given in Tables IV.1 and IV.2. Before treatment, 15 patients were *H.pylori* positive by culture and three by MG in any of the specimens. After treatment, the corpus specimens of nine patients were still positive by culture. When *H.pylori* was detected by Gram stain, it was also positive by culture. In one corpus, one antral and four duodenal biopsies, *H.pylori* was not detected by Gram stain whereas culture was found to be positive.

Table IV.1: Presence (+) or absence (-) of *H.pylori* by culture, MG, IMM and Gram stain in corpus, antrum and duodenum before therapy

Patient	corpus				antrum				duodenum			
	Cul	MG	IMM	Gr ¹	Cul	MG	IMM	Gr	Cul	MG	IMM	Gr
1	+	-	-	+	+	+	+	+	+	-	+	+
2	+	+	+	+	+	+	+	+	+	+	+	-
3	-	+	-	-	-	+	-	-	-	-	-	-
4	-	+	+	-	-	+	+	-	-	-	-	-
5	+	+	+	+	+	+	+	+	+	-	+	+
6	+	+	+	+	+	+	+	+	-	-	+	-
7	+	-	+	+	+	+	+	+	+	-	-	+
8	+	-	+	+	+	+	+	+	+	-	-	+
9	+	-	+	+	+	+	+	+	+	+	+	+
10	+	-	-	+	+	+	+	+	+	-	-	-
11	+	+	+	+	+	+	+	+	+	+	+	-
12	+	-	-	+	+	+	+	+	+	+	-	+
13	+	+	+	+	+	+	+	+	+	+	+	+
14	-	-	-	-	-	+	-	-	-	-	-	-
15	+	+	+	+	+	+	+	+	+	-	+	+
16	+	+	+	-	+	+	+	+	+	+	+	+
17	+	+	+	+	+	+	+	+	+	-	-	-
18	+	+	+	+	+	+	+	+	+	-	-	+

1 Gr: Gram stained smears of biopsy for culture

As shown in Table IV.1, before therapy, culture positive antral biopsies were also found to be positive by MG and IMM, but *H.pylori* was not detected in six corpus and eight duodenal biopsies by MG and in three corpus and six duodenal biopsies by IMM. Non-*H.pylori* was cultured from three and two of the corpus biopsies and in one duodenal biopsy when *H.pylori* was not detected by MG and IMM, respectively.

Table IV.2: Presence (+) or absence (-) of *H.pylori* by culture, MG, IMM and Gram in corpus, antrum and duodenum after therapy

Patient	corpus				antrum				duodenum			
	Cul	MG	IMM	Gr	Cul	MG	IMM	Gr	Cul	MG	IMM	Gr
1	+	+	+	+	+	-	-	+	-	-	-	-
2	+	-	-	+	+	+	+	+	+	-	-	+
3	+	-	-	+	+	+	+	+	-	+	-	-
4	-	+	-	-	-	+	-	-	-	+	-	-
5	-	-	-	-	-	+	-	-	-	-	-	-
6	+	+	+	+	-	-	-	-	-	-	-	-
7	-	-	-	-	-	-	-	-	-	+	-	-
8	+	-	+	+	-	-	-	-	-	-	-	-
9	-	-	+	-	-	+	-	-	-	-	-	-
10	+	+	+	+	+	+	+	+	-	-	-	-
11	-	-	-	-	-	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-	-	-	-	-	-
13	+	-	+	+	+	-	+	-	-	-	-	-
14	-	-	-	-	-	-	-	-	-	-	-	-
15	-	-	-	-	-	+	-	-	-	+	-	-
16	+	+	+	+	+	+	+	+	-	+	-	-
17	+	-	+	+	+	-	+	+	-	-	-	-
18	-	-	-	-	-	-	-	-	-	+	-	-

After treatment (Table IV.2) using microscopy, *H.pylori* was not detected by MG and IMM in five and two corpus, three and one antral, and one and one duodenal biopsies, respectively, whereas culture of these specimens yielded *H.pylori*. Moreover, non-*H.pylori* flora was cultured from all but one corpus biopsy.

In contrast biopsies *H.pylori* negative by culture after therapy, scored positively by microscopy, i.e. one corpus biopsy was IMM positive and one corpus, four antral and six duodenal biopsies were MG positive. Non-*H.pylori* flora was cultured in all of them except for two antral biopsies.

Before therapy active inflammation was found in antral and/or corpus biopsies from 17 patients. One patient (no. 3) showed chronic inflammation only. After therapy, active inflammation was still found in antral or corpus biopsies from seven of nine patients positive for *H.pylori* by culture. Two patients (no. 1 and 13) showed chronic inflammation. Of the nine patients with biopsies culture negative for *H.pylori*, one patient showed active inflammation in corpus (no. 14), in one patient no inflammation was found (no. 7), and seven showed chronic inflammation in antrum and/or corpus. Of these seven patients, the numbers 4, 5, 9, 15, and 18 were scored positive by histology but *H.pylori*-negative by culture. Only slight chronic inflammation was found in the antrum and corpus of no.18 and in antrum only of the others while no inflammation was found in the corpus, supporting that these were true culture-negatives.

Discussion

Although the number of patients in this study was relatively low the results showed that ongoing gastric acid inhibition is a confounding factor for the accurate histological diagnosis of *H.pylori* in gastric biopsies when a non-specific staining method is used. Because of the low eradication rate (55%) not more than 18 patients were included in this study.

At low gastric pH, before therapy, non-*H.pylori* flora is cultured in only minor amounts and use of MG does not result in many false positive *H.pylori* scores. However, after eradication therapy for *H.pylori*, many clinicians continue to treat their patients with gastric acid inhibitors for a prolonged period of time until symptoms have disappeared and/or until successful *H.pylori* eradication has been verified by control biopsies^{6,15}. In the patient population studied, ongoing gastric acid inhibition resulted in a median fasting gastric pH of 7.0 (range 1.0-7.0), only in four of the 17 patients was the gastric pH less than 3.5. As expected culture of the gastric specimens revealed other bacteria (mainly oropharyngeal flora) in addition to *H.pylori*. Similar situations may occur when *H.pylori* positive subjects have been treated with gastric acid inhibition before *H.pylori* diagnosis and in patients with atrophic gastritis. As decreased gastric acidity increased the presence of other bacterial flora in the gastric lumen and in the mucosa of corpus, antrum and duodenum, the accurate histological detection of remaining *H.pylori*

was rendered more difficult. After therapy, *H. pylori* was not detected by MG (i.e. false negative) in about half of the 17 culture positive specimens. However, with a specific immunohistochemical staining method only three of the positive specimens were missed. Also false positive results were much more frequently observed by MG (11 times) than by IMM (once), especially when non-*H. pylori* flora was present by culture. It is very likely that this flora may lead to both false positive and false negative results when using only a non-specific staining method for the histological detection of *H. pylori*. Better results are obtained by IMM compared to MG.

Recently, our culture procedures for *H. pylori* have been optimized¹¹. Washing and grinding of the specimens was carried out to detect low amounts of *H. pylori* and to reduce contaminating oropharyngeal flora. In addition, Skirrow selective medium was used for optimal isolation of *H. pylori* in combination with the lowest rate of non-*H. pylori* flora. Therefore, in the present study culture was used as a standard to compare the usefulness of IMM and MG for the detection of *H. pylori*. Furthermore, *H. pylori* culture results were always positive when Gram stained smears of the biopsies were positive. Only in one patient before therapy (no. 4) were biopsy specimens of antrum and corpus were positive both by IMM and MG but not by culture. This single exception does not influence the conclusions concerning the usefulness of MG and IMM for the detection of *H. pylori*.

Surprisingly, the histological detection of *H. pylori* often failed (both by IMM and MG) in the duodenal biopsies in comparison with culture results. A possible explanation might be the use of a homogeneous sample of a whole ground biopsy for culture, whereas only sections of a biopsy are used for histology. This may particularly influence detection in duodenal biopsies containing low amounts of *H. pylori*.

The specificity of the polyclonal antiserum used was tested and no cross reactivity was found with *Campylobacter jejuni*, *Campylobacter coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Streptococcus viridans* and *Staphylococcus aureus*. In addition forty different *H. pylori* strains isolated from different patients were found to be positive. Comparing the same *H. pylori* antiserum in an immunohistochemical stain with a cresyl-violet stain (comparable to the modified Giemsa stain), Navratil et al.¹⁶ found a high sensitivity and specificity for both stains after eradication therapy for *H. pylori* with a slightly higher sensitivity of the IMM stain. However, patients in that study had been off gastric acid inhibition (lansoprazole) more than four weeks before re-testing and it is to be expected that gastric pH had returned to normal.

The histological diagnosis of *H. pylori* in patients with ongoing gastric acid

inhibition therapy will remain unreliable if only non-specific staining methods (such as the modified Giemsa) are used. In this common clinical situation, false positive and false negative histological detection of *H.pylori* may occur. The use of a highly specific immunohistochemical stain is strongly recommended for the histological diagnosis of *H.pylori* to prevent unnecessary repetition of eradication therapy.

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Chapter V

**The gastroduodenal distribution
of *Helicobacter pylori* in
patients with duodenal and
gastric ulcer:
relation to inflammatory
changes, mucosal atrophy and
intestinal metaplasia in the
stomach and gastric metaplasia
in the duodenum**

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Summary

The relation between *H.pylori* infection, inflammation, mucosal atrophy, intestinal and gastric metaplasia was studied in 49 patients with a duodenal ulcer (DU) (median age 56 years, 33M/16F) and 47 patients with a gastric ulcer (GU) (median age 67 years, 22M/25F). At least two biopsy specimens of duodenal bulb, antrum and corpus mucosa were obtained for histology. Serial sections were stained by modified Giemsa (MG) and Immunohistochemistry (IMM) (DAKO B471) for the detection of *H.pylori*. Bacterial density was graded semi-quantitatively. Haematoxylin-Eosin (HE) stained sections were used for the histological classification of chronic and active inflammation, mucosal atrophy and intestinal metaplasia. An additional section of duodenal bulb was stained with the PAS/Alcian blue to assess the presence of gastric metaplasia.

H.pylori was found in 81.6% cases of DU and in 83.0% cases of GU. In DU and GU patients, *H.pylori* was found in 79.6% and 79.5% of corpus, 77.6% and 72.3% of antral, and 16.7% and 20.0% of duodenal bulb mucosal biopsies, respectively. In DU patients, the density of *H.pylori* was significantly lower in corpus compared to antrum biopsies ($p < 0.001$). No such difference was observed in GU patients. Furthermore, the *H.pylori* density was higher in corpus of GU than of DU patients. Inflammatory scores followed the density of *H.pylori* infection. Significantly higher prevalences of mucosal atrophy and intestinal metaplasia were found in GU than DU patients ($P < 0.05$). The occurrence and extent of gastric metaplasia were comparable in the duodenal bulb of both DU and GU patients.

In conclusion, some differences were found in the gastroduodenal distribution of *H.pylori* in DU and GU patients which may contribute to differences in presentation of the both conditions. The higher prevalence of precancerous lesions (atrophy and intestinal metaplasia) in GU patients will be associated with the higher risk for gastric cancer. No evidence was found for a role of gastric metaplasia in the differential pathogenesis of DU and GU. It is probable that also other bacterial factors (such as differences in *H.pylori* isolates), host factors, and environmental factors play a role in the differentiation between the two forms of peptic ulcer disease.

Introduction

Helicobacter pylori (*H.pylori*) can be found in 90%-100% of patients with duodenal ulcer (DU)¹⁻⁶ and in 60%-100% of patients with gastric ulcer (GU)^{1,3,4,6,7}. Eradication of *H.pylori* results in a significant reduction of both DU^{8,9} and GU recurrence¹⁰⁻¹² suggesting an important - possibly causative - role of *H.pylori* in the pathogenesis of DU and GU.

H.pylori has several properties which can induce tissue damage: production of ammonia resulting from its extreme urease activity, production of cytotoxins, and induction of local and humoral inflammatory responses^{6,13,14}. In addition, there are other possible pathogenetic mechanisms: DU patients and healthy volunteers with *H.pylori* infection have higher basal and stimulated plasma gastrin concentrations and higher basal and stimulated acid output compared to those who are negative for *H.pylori*¹⁵⁻¹⁷. Also pepsinogen I and II concentrations are higher in *H.pylori* positive subjects¹⁶.

Kozol et al.² describes four mechanisms by which *H.pylori* may contribute to the pathogenesis of duodenal ulceration: First, *H.pylori* may colonize areas of gastric metaplasia in the duodenum and cause a duodenal ulcer by local mucosal damage. A second mechanism results from an increased acid secretion and hypergastrinaemia associated with *H.pylori*. Gastrin release could be stimulated by local pH increase in the gastric mucosa by the production of ammonia through the urease activity, or by a direct effect of inflammatory mediators on gastrin producing cells. Increased intragastric acidity has been associated with the development of gastric metaplasia¹⁸. Thirdly, the duodenal mucosa could be damaged by passage of inflammatory mediators from the gastric lumen into the duodenum. Finally, the duodenal mucosa may be directly damaged by an immune response to *H.pylori*. These mechanisms are not necessarily mutually exclusive. The aetio-pathogenetic role of *H.pylori* in GU patients has hardly been discussed.

Before the *H.pylori* era, many other factors were found to contribute to the development of DU and GU, such as genetic predisposition, diet, smoking and psychosocial factors. However, the precise pathophysiological mechanisms and differences of such mechanisms in DU and GU are not clear yet.

In this study, DU and GU patients were compared in terms of gastroduodenal distribution and density of *H.pylori*, prevalence of precancerous lesions of the gastric mucosa (atrophy and intestinal metaplasia) and the prevalence of gastric metaplasia in the duodenal bulb, in order to look for differences between both patients groups that may contribute to the differences in the presentation of peptic ulcer disease.

Material and methods

Patients and specimens

Specimens were obtained from patients with newly diagnosed duodenal or gastric ulcers during open access endoscopy. Prior to gastroscopy, four DU and two GU patients used H₂-antagonists and one DU and one GU patient were using omeprazole. NSAIDs were used by five DU and six GU patients. At the endoscopy, the pH of gastric juice was measured using pH paper strips with grading steps of 0.5 from pH 0 to 14 (Schleicher & Schüll GmbH; Dassel, Germany).

At least two adjacent biopsies were taken from corpus, antral and duodenal bulb mucosa for histology using an Olympus GIFQ gastrocope. Biopsies were fixed in formalin and embedded in paraffin; serial sections of 4 µm were cut and stained by Haematoxylin-Eosin (HE), modified Giemsa (MG)¹⁹ and an immunohistochemical stain (IMM) against *H.pylori* using a purified polyclonal antiserum (DAKO B471, ITK Diagnostics BV, Uithoorn, The Netherlands)²⁰. Duodenal biopsies were also stained with the periodic acid Schiff (PAS)/Alcian blue stain.

Detection of *Helicobacter pylori*

The MG and IMM slides were scored without knowledge of the clinical diagnosis for the presence of *H.pylori* at 400 magnification and graded 0-3: no *H.pylori* found (grade 0); occasionally *H.pylori* found after searching (grade 1); scattered *H.pylori* bacteria in many fields (grade 2); numerous *H.pylori* bacteria in most fields (grade 3)^{1,21}. The presence of *H.pylori* by MG and IMM was characterised by the S-shaped appearance of the bacteria in MG or by immunoreactivity in IMM and by their localisation in the gastric mucus layer as well as in the gastric pits. At least two cross-sections of all biopsies were examined completely. Special attention was paid to the presence of non-*H.pylori* bacteria through assessment of morphology and localisation of microorganisms in the MG stain in combination with absent immunoreactivity in the identical area of a consecutive slide.

Histological findings in corpus and antrum

HE stained corpus and antrum sections were scored for the presence of chronic inflammation, neutrophil activity, intestinal metaplasia and mucosal atrophy according to the Sydney classification²². Duodenal bulb biopsies were scored for the presence of chronic and active inflammation on HE stained sections. The extent of gastric antral metaplasia was assessed with the PAS/Alcian blue stain using a

scale of 0%-100% involvement of the epithelial layer present in the biopsies. The score was based on mutually agreed values of two observers (who were not aware of the diagnosis of DU or GU) and divided into four grades: no gastric metaplasia present (grade 0); 10% or less (grade 1); between 10% and 50% (grade 2); 50% of more gastric type epithelium present (grade 3).

Statistical analysis

Age and intragastric pH of DU and GU patients were compared with a Mann-Whitney U test. Prevalences of *H.pylori* positive duodenal, antral and corpus mucosal biopsies and prevalence of chronic and active inflammation, mucosal atrophy, and intestinal metaplasia were analyzed between and within DU and GU patients using a Chi-Square test and a McNemar test, respectively. Differences in density of *H.pylori* were analyzed with a Mann-Whitney U test between DU and GU patients and with a Wilcoxon test within DU and GU patients. The prevalence and grading of gastric metaplasia in the duodenal bulb of DU and GU patients were analyzed using a Chi-Square test and a Mann Whitney U test, respectively. A p -value < 0.05 was considered to be significant.

Results

Patients

Biopsy specimens were obtained from 49 DU (33 male and 16 female) and 47 GU (22 male and 25 female) patients. Biopsies from corpus, antrum and duodenum were available of 48 DU and 42 GU patients. Duodenal biopsies were missing from 1 DU and 2 GU patients and corpus biopsies were missing from 3 GU patients. In most cases two or more biopsies from each site were available, except for 6 times antral, 4 times corpus and 2 times duodenal sampling (from 7 DU and 3 GU patients). Median age for DU was 56 (21-85) years and for GU 67 (34-93) years ($p < 0.01$). The median pH of gastric juice was 1.5 (1.0-7.0) for DU and 1.5 (1.0-7.5) for GU patients with a pH above 3.5 in 5 of 43 (11.6%) DU and in 14 of 39 (35.9%) GU patients ($p < 0.01$). After exclusion of the few patients treated with gastric acid inhibitors, still a significantly higher intragastric pH was found in GU as compared to DU patients ($p < 0.05$).

Prevalence and density of *Helicobacter pylori*

H.pylori was detected by IMM in 40/49 (81.6%) DU and 39/47 (83.0%) GU patients. Comparing the presence of *H.pylori* according to IMM and MG stains,

respectively, minor but not significant differences in detection were found. Concordance between both stains was 98.0% for DU and 89.4% for GU patients. Non-*H.pylori* bacteria were detected in 5 DU (10.2%) patients and in 8 GU patients (17.0%). Because of the higher sensitivity and specificity of IMM compared to MG for the detection of *H.pylori*²⁰, the results of IMM were used for further analyses.

The prevalence of *H.pylori* in corpus, antrum or duodenum of DU patients was not statistically different from GU patients (Table V.1 and V.2). Neither were any differences observed in *H.pylori* infestation observed between antrum and corpus biopsies within the group of DU and GU patients (Table V.2). *H.pylori* prevalences were found with a similar frequency in duodenum of both DU and GU patients and were encountered significantly less frequently than in the antrum or corpus of these patient groups ($p < 0.001$).

Table V.1: Presence (%) of *H.pylori*, chronic and active inflammation, intestinal metaplasia and mucosal atrophy scores in corpus, antrum and duodenum of DU and GU patients

Histology	corpus	antrum	duodenum
DU patients	n=49	n=49	n=48
<i>H.pylori</i> (IMM)	39 (79.6%)	38 (77.6%)	8 (16.7%)
chron inflamm	30 (61.2%)	42 (85.7%)	27 (56.3%)
act inflamm	9 (18.4%)	31 (63.3%)	9 (18.8%)
intest metapl	1 (2.0%)	16 (32.7%)	- (0.0%)
mucosal atrophy	1 (2.0%)	9 (18.4%)	- (0.0%)
GU patients	n=44	n=47	n=45
<i>H.pylori</i> (IMM)	35 (79.5%)	34 (72.3%)	9 (20.0%)
chron inflamm	34 (77.3%)	44 (93.6%)	19 (42.2%)
act inflamm	20 (45.5%)	35 (74.5%)	5 (11.1%)
intest metapl	9 (20.5%)	25 (53.2%)	- (0.0%)
mucosal atrophy	8 (18.2%)	13 (27.7%)	- (0.0%)

For DU patients the density of *H.pylori* was significantly higher in antral than in corpus biopsies ($p < 0.001$) (Figure V.1a). Similar densities were found in antrum and corpus of GU patients (Figure V.1b). Comparing DU with GU

patients, no significant differences in the *H. pylori* density were found in antrum but a significantly higher *H. pylori* density was found in corpus of GU than of DU patients ($p < 0.01$) (Table V.2). The *H. pylori* density in duodenal bulb biopsies was significantly higher in DU than GU patients ($p < 0.05$). However, these results are based on small numbers.

Figure V.1: Density of *H. pylori* positive corpus, antral and duodenal biopsies of DU patients (A) and GU patients (B)

Histological findings in the stomach and their relation to *Helicobacter pylori*

In both DU and GU patients, chronic and active gastritis were significantly more frequent in antrum than corpus. This difference was more pronounced for DU than GU patients (Table V.1 and V.2). Although antrum gastritis occurred to a similar degree in DU and GU patients, GU patients more frequently showed chronic ($p < 0.05$) and active inflammation ($p < 0.01$) in the corpus (Table V.1 and V.2).

Table V.2: Statistical differences in prevalence and density of *H. pylori* and the presence of inflammation (chronic and active), intestinal metaplasia and mucosal atrophy between and within DU and GU patients

	DU patients		GU patients
<i>H. pylori</i> prevalence	antrum	=	antrum
	=		=
	corpus	=	corpus
<i>H. pylori</i> density	antrum	=	antrum
	> ***		=
	corpus	< **	corpus
Chronic inflammation	antrum	=	antrum
	> **		> *
	corpus	< *	corpus
Active inflammation	antrum	=	antrum
	> ***		> **
	corpus	< **	corpus
Intestinal metaplasia	antrum	< *	antrum
	> ***		> ***
	corpus	< **	corpus
Mucosal atrophy	antrum	=	antrum
	> **		=
	corpus	< *	corpus

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

Intestinal metaplasia and mucosal atrophy were found in both DU and GU patients. Intestinal metaplasia was more frequently in antrum than corpus of both groups ($p < 0.001$), and significantly more often in GU than in DU patients ($p < 0.05$) (Table V.1 and V.2). DU patients with intestinal metaplasia in the antrum (median age 68 (range 37-79) years) were significantly older than those without intestinal metaplasia (median age 45 (range 21-85) years) ($p < 0.01$). Mucosal atrophy occurred more often in antrum and corpus of GU patients than of DU patients, although the difference was significant for the corpus only ($p < 0.05$). In both patient groups mucosal atrophy was found more frequently in antrum than corpus, being significant only for DU patients ($p < 0.01$). In GU patients, the presence of intestinal metaplasia and mucosal atrophy in the corpus was associated with a significantly higher intragastric pH ($p < 0.01$), also after exclusion of the patients treated with acid inhibitors.

Relating the *H.pylori* status to mucosal findings, in DU patients the presence of *H.pylori* correlated with the presence of chronic and active gastritis in antrum and in GU patients with the presence of chronic gastritis in corpus and active gastritis in antrum. In all patients, *H.pylori* was significantly less frequently found in the presence of intestinal metaplasia and mucosal atrophy ($p < 0.05$). Furthermore, in both DU and GU patients the *H.pylori* density in corpus was significantly higher when mucosal atrophy or intestinal metaplasia was present in the antrum ($p < 0.05$).

Histological findings in duodenal bulb and the relation to *Helicobacter pylori*

Chronic and active inflammation were found in the duodenal bulb of 56.3% and 18.8% of DU and 42.2% and 11.1% of GU patients, respectively (Table V.1). *H.pylori* was detected in the duodenal bulb of eight DU (16.7%) and of nine GU patients (20.0%). In DU patients, *H.pylori* was found in combination with chronic inflammation in all eight patients and in combination with activity in only two patients. No association of *H.pylori* with chronic active inflammation was found in GU patients.

Table V.3: Presence of gastric metaplasia (grade 1,2,3) in duodenal bulb biopsies of DU and GU patients

	n	positive	grade 1	grade 2	grade 3
DU	45	18 (40.0%)	7 (38.9%)	5 (27.8%)	6 (33.3%)
GU	43	14 (32.6%)	9 (64.3%)	4 (28.6%)	1 (7.1%)

No significant differences were found in either the prevalence or the degree of gastric metaplasia in both DU and GU patients, although the latter appeared slightly higher in DU patients (Table V.3). Furthermore, no correlation could be found between the presence of gastric metaplasia and the presence of chronic or active inflammation or between gastric metaplasia and the intragastric pH in both patient groups. Gastric metaplasia was present in 5 of 8 *H.pylori* positive DU patients and in 3 of 9 *H.pylori* positive GU patients (Figure V.2).

Figure V.2: Presence of *H.pylori* and gastric metaplasia in duodenal biopsies of DU and GU patients

Helicobacter pylori negative patients

Of the nine *H.pylori* negative DU patients, one patient used NSAID, and one paracetamol, but seven had not used any medication within three months prior to the gastroscopy. Of the eight *H.pylori* negative GU patients, one patient used omeprazole before endoscopy, two used NSAID, and five patients did not use any medication.

In 2 of 9 *H.pylori* negative DU patients intestinal metaplasia was found in the

antrum, once in combination with mucosal atrophy in the antrum. In 4 of 8 GU patients intestinal metaplasia was found in both antrum and corpus: once with mucosal atrophy in the corpus and once with mucosal atrophy in the antrum also.

Discussion

In this study *H.pylori* infection occurs with a similar frequency in DU and GU patients in both antrum and corpus as described by Madsen et al.²¹, whereas Louw et al.⁴ find a higher *H.pylori* frequency in antrum of the DU patients. In line with the latter and with other reports^{5,23}, in DU patients a higher density of *H.pylori* in antrum compared to corpus was observed and a higher density in corpus of GU than DU patients. In line with these findings a predominantly antral gastritis was found in DU patients^{4,21,24,25} and a more widespread intragastric inflammatory response^{4,25} with higher prevalences of mucosal atrophy and intestinal metaplasia was seen in GU patients. Finally, comparably low prevalences of *H.pylori* and gastric metaplasia were present in the duodenal bulb of both DU and GU patients.

To our knowledge this is the first study in which gastroduodenal bacterial flora of DU and GU patients was studied simultaneously with the histochemical MG stain and a *H.pylori* specific immunohistochemical stain. In peptic ulcer disease, there seems to be a good correlation between both stains. In this study, a non-*H.pylori* bacterial flora was only found in few ulcer patients. In the five DU patients, no association was found between the presence of non-*H.pylori* flora and mucosal atrophy or intestinal metaplasia. In GU patients, the non-*H.pylori* bacteria were present in 6 of 8 patients in combination with intestinal metaplasia and/or atrophy, found in the antrum only of 2 patients, in antrum and corpus of 3 patients and in corpus only of 1 patient. This indicates that especially in the GU patients these precancerous lesions create an environment where non-*H.pylori* bacterial flora can colonize.

The prevalence of *H.pylori* in DU and GU patients observed by us are in line with findings of others⁶, although the prevalence in DU patients is in the lower range. The absence of *H.pylori* in 9 DU patients cannot fully be explained by the use of medication prior to gastroscopy or by the presence of atrophy and intestinal metaplasia. Previously, also others have described a similar proportion of *H.pylori* negative PUD patients^{26,27}.

Several host or environmental factors may contribute to the differences between DU and GU in prevalence and density of *H.pylori* and inflammation in antrum and corpus. First, a higher density may reflect the duration or chronicity of the *H.pylori* infection⁶: GU patients were older at time of diagnosis and the density

in corpus biopsies was higher than in DU patients. This could advocate for an ascension of the infection with increasing age. Secondly, there may be a difference in affinity of *H.pylori* to the mucosa or in adherence factors with possible differences over antrum and corpus⁴. The availability of nutrients for *H.pylori* in antrum or corpus may differ between patients groups⁴. Madsen et al.²¹ and Bayerdörffer et al.²⁴ also mention differences in mucosal reactivity towards *H.pylori* between antrum and corpus.

Furthermore the intragastric pH and precancerous lesions can influence the *H.pylori* status. *H.pylori* is generally more frequently found in the non-acid secreting antrum^{5,24}. The lower gastric acidity present in GU compared to DU patients may promote the infection with *H.pylori* of the more proximal parts of the stomach²⁸.

Finally, the density of *H.pylori* can be influenced by the presence of mucosal atrophy and intestinal metaplasia^{29,31}; indeed in this study, the prevalence of *H.pylori* was negatively associated with the presence of both histological lesions. However, from a transversal study like this, it cannot be concluded whether the latter two factors are a cause or a consequence of differences in density of *H.pylori*.

Although mucosal atrophy and intestinal metaplasia were found in both DU and GU patients, they were most frequently seen in GU patients as reported by Louw et al.⁴. Both mucosal atrophy and intestinal metaplasia are associated with an increased risk of gastric cancer, especially of the intestinal type³²⁻³⁵. This supports the reports of Correa et al.³⁶ and Hansson et al.³⁷, indicating that GU patients are at higher risk to develop gastric cancer compared with DU patients.

Gastric metaplasia is reported in 50-100% of DU patients^{5,38-40}. In GU patients, a lower frequency is reported of 19%³⁹. Some authors have described that *H.pylori* is only present in duodenal areas with gastric metaplasia^{5,17,38-43} and that subsequent duodenal inflammation and ulceration leads to mucosal damage⁵. A second hypothesis suggests that *H.pylori* infection of the gastric antrum increases the gastric acid production, leading to a greater duodenal acidity¹⁷ which induces gastric metaplasia and duodenal damage independent from each other.

In this study the overall frequency of gastric metaplasia was low in both DU and GU, although at least two duodenal biopsies of the first part of the duodenum were evaluated as recommended by Wyatt et al.¹⁸. Furthermore, the prevalence of gastric metaplasia was similar in GU patients who did not have duodenal lesions. Therefore gastric metaplasia cannot be a prerequisite for DU formation.

Several studies have shown that there was no concordance between the presence of gastric metaplasia and *H.pylori*^{25,40}; in many specimens with gastric metaplasia no *H.pylori* could be detected. This is in agreement with our findings

and advocates against a close pathogenetic link between gastric metaplasia in the duodenal bulb, *H.pylori* infection and initiation of DU. In his review Kozol et al.² come to the same conclusion because only low duodenal frequencies of *H.pylori* are reported in the literature. They suggest that the release of inflammatory mediators into the gastric lumen that washdown to the duodenum may play a role.

In conclusion, there is a different pattern of *H.pylori* colonization and mucosal inflammation, intestinal metaplasia and mucosal atrophy in the both subtypes of peptic ulcer disease. However, our study does not allow to draw conclusions as to whether the differences of *H.pylori* colonization are a cause of these different histological patterns or a consequence of predetermined changes in the gastric mucosal lining. Longitudinal study of bacterial factors and host factors will hopefully elucidate the role of *H.pylori* in the differential pathogenesis of GU and DU. However, gastric metaplasia in the duodenum with or without *H.pylori* infection seems not to play a major part in DU pathogenesis. The higher degree of mucosal atrophy and of intestinal metaplasia found in GU patients support the finding of a higher risk for gastric cancer in these patients as it is previously described by Hansson et al.³⁷.

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Chapter VI

***Helicobacter pylori* and non-*Helicobacter pylori* bacterial flora in gastric mucosal and tumour specimens of patients with primary gastric lymphoma**

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Summary

There is an association of *Helicobacter pylori* (*H.pylori*) with gastric mucosa-associated lymphoid tissue (MALT) and MALT-lymphoma. Histologically, mainly non-specific stains are used to detect *H.pylori*, such as Haematoxylin-Eosin (HE) or modified Giemsa (MG).

In this study both a MG and a specific immunohistochemical stain (IMM) for *H.pylori* (DAKO B471) were performed on sequential slides of resection material containing tumour and non-tumorous gastric mucosa from patients with primary gastric lymphoma (n=52). Special attention was paid to the presence of non-*H.pylori* bacterial flora diagnosed by a positive MG (according to form and localisation) and a subsequently negative IMM. On all slides bacterial density was scored semi-quantitatively (grades 0,1,2,3).

In total 32 (61.5%) patients were *H.pylori*-positive by IMM and 34 (65.4%) were non-*H.pylori* positive by MG. In 24 of the 34 patients the non-*H.pylori* flora consisted mainly of cocci, in combination with rods in 15 patients, mostly in minor quantities; in another ten patients high numbers of both cocci and different types of rods were present. Most non-*H.pylori* bacteria were localized superficially, although in 22 patients minor quantities of non-*H.pylori* were seen in the glandular lumina also. Analysing all patients, no differences in density of *H.pylori* and of non-*H.pylori* flora were found. Only when comparing patients with a small to those with a large cell lymphoma, a significantly higher density of *H.pylori* was found in the corpus mucosa of large cell lymphomas and a higher prevalence of non-*H.pylori* was found in tumour, in antrum or corpus, of patients with large cell lymphomas.

In conclusion, using joint evaluation by modified Giemsa and a *H.pylori*-specific immunohistochemical stain, the proportion of *H.pylori* positive gastric lymphoma patients was lower than in most previous publications and other bacteria were found in a relatively high proportion. The role of the non-*H.pylori* intragastric bacterial flora identified in this study has to be further elucidated with respect to the aetio-pathogenesis of primary gastric lymphoma.

Introduction

Helicobacter pylori (*H.pylori*) has been found in the gastric non-tumorous mucosa of 50% to more than 90% of patients with primary gastric lymphoma¹⁻⁴. A serological study of Parsonnet⁵ has shown that patients with gastric lymphoma have a significantly increased prevalence of previous *H.pylori* infection compared to matched controls. Furthermore, first results on eradication of *H.pylori* have demonstrated regression or disappearance of primary low grade gastric MALT lymphoma⁶⁻⁸.

In patients with gastric cancer an increased intragastric pH with an increased risk of bacterial overgrowth has been observed⁹⁻¹¹. Metabolic products of bacteria such as N-nitroso compounds, may contribute to the pathogenesis of the malignant process. It might be possible that a similar situation of reduced acidity and bacterial overgrowth occurs in patients with primary gastric lymphoma.

In histological studies of patients with primary gastric (MALT) lymphoma generally non-specific staining methods were used for the detection of *H.pylori* such as the Haematoxylin-Eosin or modified Giemsa stains^{1,3,4}. In a previous study, we found a high specificity and sensitivity of an immunohistochemical stain for *H.pylori*¹², which has been confirmed by others¹³.

In this study we wanted to assess the presence and distribution of the bacterial flora in gastric tissue specimens of patients with primary gastric lymphoma by microscopy using a modified Giemsa stain for the total bacterial flora (i.e. *H.pylori* and non-*H.pylori* bacterial flora), combined with a specific immunohistochemical stain for the detection of *H.pylori*.

Material and methods

Patients and specimens

Fifty-two patients (mean age 62.4 years; 31 male and 21 female) from ten hospitals in the provinces of Limburg and Brabant in the Netherlands with a documented primary gastric B-cell lymphoma were included in the study. For inclusion in this study, both tumour tissue and non-tumorous gastric mucosa had to be present in the surgically resected specimens (n=50) or endoscopic biopsies (n=2).

Histology

Serial sections of 4 μ m from formalin fixed and paraffin embedded specimens were cut for Haematoxylin-Eosin stain (HE), modified Giemsa stain (MG) and an

immunohistochemical stain (IMM), respectively. HE stained sections were revised for the classification of the primary gastric lymphoma and to assess the anatomical tissue type (i.e. antral or corpus type) of the mucosa present in the specimens, which were used for the assessment of *H.pylori* and other bacteria. MG was performed as described by Gray et al.¹⁴ to discover intragastric bacteria. For the IMM, a purified polyclonal antibody against *H.pylori* (DAKO B471, ITK Diagnostics BV, Uithoorn, The Netherlands) was applied. The antiserum was used in a 1:500 dilution. Further staining was performed with biotin-conjugated swine-anti-rabbit-antibodies (1:1000) (DAKO E431) as a second layer, followed by streptABC-HRP (1:1000) (DAKO K377). Sections were then stained with diaminobenzidine with 10% imidazol to intensify the reaction and were counter-stained with haematoxylin.

In the MG stain, the presence of *H.pylori* was scored according to the S-shaped form and the localisation on or within the mucus layer as well as within the crypts. The presence of *H.pylori* was only accepted if IMM on consecutive slides was positive. The presence of non-*H.pylori* flora was suspected in the MG stain by form and localisation of the bacteria and confirmed by negativity in the IMM.

All slides were examined completely at 400x magnification. If necessary a 1000x magnification was used. Both *H.pylori* and non-*H.pylori* were scored semi-quantitatively and graded as follows: no bacteria found, grade 0; occasionally bacteria found, grade 1; scattered bacteria found in many high power fields, grade 2; and numerous bacteria found in most high power fields, grade 3¹⁵.

Analysis

Prevalence and density of *H.pylori* and non-*H.pylori* were compared in total and according to their presence in tumour tissue or non-tumorous antral or corpus type mucosa. Differences in prevalence were analyzed using a Chi-Square test with Fisher's Exact test when necessary. Differences in density of *H.pylori* or non-*H.pylori* positive specimens were analyzed with a Mann-Whitney U test. The prevalence and density of *H.pylori* and non-*H.pylori* in antrum compared to corpus and in tumour tissue compared to mucosa within the patients, were analyzed by paired non-parametrical tests as well.

Differences in the sex distribution and lymphoma type (small cell versus large cell gastric lymphoma) between different patient subgroups were analyzed with the Chi-Square test. Differences in age between the different groups were compared using the Mann-Whitney U test or Kruskal-Wallis one-way Anova, depending on the number of groups to be compared.

A p-value of less than 0.05 was considered to be significant.

Results

Of 52 patients included, 17 had a small cell and 35 a large cell primary gastric lymphoma according to the criteria given by Isaacson^{16,17}. The diagnosis of MALT could be confirmed in 43 of 52 patients. The remaining nine large cell lymphomas could not further be classified as being from MALT origin because of the absence of a small cell component. Besides tumour tissue, normal appearing antral or corpus mucosa, or both types of mucosa were present for 9, 8, and 35 patients, respectively.

Prevalence of *Helicobacter pylori* and non-*Helicobacter pylori* per patient

H.pylori could be detected by IMM in 32 of the 52 (61.5%) patients. Using MG 36 of 52 (69.2%) patients were scored *H.pylori* positive. Non-*H.pylori* flora was detected in 34 of 52 (65.4%) patients by MG. *H.pylori* and non-*H.pylori* were present simultaneously in 19 of 52 (36.5%) patients, *H.pylori* only in 13 (25.0%) and non-*H.pylori* flora only in 15 patients (28.8%). In five (9.6%) patients no bacteria were found in any of the tissue specimens. *H.pylori* was only detected in the non-tumorous gastric mucosa present in the specimens, in contrast to non-*H.pylori* flora which was found on and within superficial tumour tissue as well as in the surrounding gastric mucosa.

For further analysis as to the prevalence and density of *H.pylori*, IMM results were considered as the standard for the detection of *H.pylori* because of the proven high specificity and sensitivity of IMM^{12,13}.

Description of non-*Helicobacter pylori* flora

In 24 of 34 patients the non-*H.pylori* flora consisted mainly of cocci, 15 times in combination with rods. The density of cocci was higher than that of rods in nine of them. In the other ten patients, massive numbers of both cocci (grade 3) and of different types of rods (grade 3) were found. When several resection specimens were available from one patient, the bacterial forms observed were similar for each patient.

In tumour tissue the non-*H.pylori* flora was localised on the surface of and within the superficial layer of the tumour. When non-*H.pylori* flora was observed in the gastric mucosa, it was mainly localised superficially (in strains of mucus overlying the mucosa), although in 22 patients minor numbers were also seen on or within the mucus layer of the glands. In four patients coccoid bacteria were also present between *H.pylori* bacteria in the mucus layer of the glands. This will be coccoid forms of *H.pylori*, as they were found to be IMM positive.

In addition to the bacteria observed, in 16 and 9 patients, respectively, yeast cells (median density grade 2) and mycelium and/or pseudo-mycelium threads (median density grade 3) (confirmed by Grocott stain) were seen, predominantly in the tumour area.

Prevalence and density of *Helicobacter pylori* and non-*Helicobacter pylori* in antrum and corpus

In Table VI.1 the prevalence of *H.pylori* (by IMM) and non-*H.pylori* (by MG) in antrum and corpus specimens is given for all patients. Figure VI.1 shows the frequency of grade 1, 2, and 3 as percentage of *H.pylori* or non-*H.pylori* positive specimens per anatomical site. Per patient the median gradation per anatomical site was used for the analyses of the density, if more than one specimen was available. When the median value was lower than the 75th percentile of the gradation, we have chosen a "worst case" approach and used the 75th percentile.

Table VI.1: Prevalences of *H.pylori* and non-*H.pylori* in antral and corpus specimens

Anatomical site		n	<i>H.pylori</i> pos	non- <i>H.pylori</i> pos
Antrum	mucosa	44	20 (45.5%)	24 (54.5%)
	tumour	36	-	19 (52.8%)
Corpus	mucosa	43	27 (62.8%)	21 (48.8%)
	tumour	35	-	18 (51.4%)

A tendency ($p=0.06$) was observed concerning a higher prevalence of *H.pylori* in corpus (62.8%) compared to antral type mucosa (43.2%). No significant differences were found in the density of *H.pylori* positive antrum or corpus specimens.

Non-*H.pylori* flora was found in antral mucosa of 24 of 44 patients (54.5%) and in corpus type mucosa of 21 of 43 patients (48.8%). No significant differences were found in either the prevalence or the density of this non-*H.pylori* flora in antral or corpus type mucosa. Moreover, both the prevalence and density of non-*H.pylori* flora were similar on or within tumour tissue in antrum (50.0%) or corpus (51.4%) compared to the normal appearing antral (54.5%) or corpus (48.8%) mucosa, respectively.

Comparing the presence of *H.pylori* with that of non-*H.pylori* in antrum or corpus, both types of flora were found with a similar frequency and density.

Among the patients with primary gastric lymphoma, four groups can be

distinguished according to the bacterial floras detected: those with *H.pylori* only (group 1, n=13), with non-*H.pylori* only (group 2, n=15), with both *H.pylori* and non-*H.pylori* (group 3, n=19) and those where no bacteria could be detected in any of the specimens (group 4, n=5).

Figure VI.1: Density of *H.pylori* and non-*H.pylori* flora in antrum and corpus mucosa or tumour tissue (Percentage of grade 1,2,3 of total number of positives)

Table VI.2: Sex distribution, age and lymphoma type (small/large cell lymphoma) of patients according to the presence of *H.pylori* (Hp) and/or non-*H.pylori* (nHp) bacterial flora

	Hp only gr 1, n=13	nHP only gr 2, n=15	both Hp/nHp gr 3, n=19	no flora gr 4, n=5
Age median (range)	56 (26-79) ¹	60 (50-92)	64 (35-83)	72 (61-87) ¹
Sex male/female	10/3 ²	6/9 ²	12/7	3/2
Lymphoma type small/large cell	7/6	3/12	6/13	1/4

¹ Sign. higher age in groups 4 versus group 1 (Mann-Whitney, $p < 0.05$)

² Sign more females in group 2 versus group 1 (Chi-Square, $p < 0.05$)

No statistical differences were observed in the distribution of small cell versus large cell gastric lymphoma between the four groups, although a trend was found for a higher number of large cell lymphomas to be present in group 2 ($p=0.07$) and group 3 ($p=0.07$) compared with group 1. The age of patients in group 4 was significantly higher than of group 2 ($p=0.04$) and in group 2 significantly more females were present compared with group 1 ($p=0.048$). No further differences concerning gender and age between the four groups were found (Table VI.2). Comparing the presence of *H.pylori* in group 1 with group 3, or the presence of non-*H.pylori* in group 2 with group 3, no significant differences were found in the prevalence or density of *H.pylori* or non-*H.pylori* in antrum or corpus. Also within each group no differences were observed comparing antrum with corpus and comparing *H.pylori* with non-*H.pylori* (Table VI.3, Figure VI.2).

Table VI.3: Prevalence of *H.pylori* and non-*H.pylori* flora in antrum and corpus of patients with *H.pylori* only, non-*H.pylori* only or both types of flora

		Hp only pos/n (%)	nHp only pos/n (%)	both Hp/nHp pos/n (%)
<i>H.pylori</i>				
antrum	mucosa	9/10 (90.0)	- -	11/15 (73.3)
corpus	mucosa	11/12 (91.7)	- -	16/17 (94.1)
non-<i>H.pylori</i>				
antrum	mucosa	- -	11/14 (78.6)	13/15 (86.7)
	tumour	- -	10/12 (83.3)	9/13 (69.2)
corpus	mucosa	- -	6/11 (54.5)	15/17 (88.2)
	tumour	- -	7/8 (87.5)	11/16 (68.8)

Figure VI.2: Density of *H. pylori* (A) or non-*H. pylori* in gastric mucosa (B) and tumour tissue (C) of patients with only *H. pylori* (group 1) or only non-*H. pylori* (group 2) compared with patients with both *H. pylori* and non-*H. pylori* (group 3)

***Helicobacter pylori* and non-*Helicobacter pylori* flora in small versus large cell gastric lymphoma**

In total 17 patients had a small (10 male/7 female, median age 55 (26-83) years), and 35 patients had a large cell gastric lymphoma (21 male/14 female, median age 67 (35-92) years). No significant differences were found in the distribution of males and females in both groups. However, patients with a large cell lymphoma were significantly older (Mann-Whitney, $p=0.03$) than those with a small cell lymphoma.

Table VI.4: Prevalence (%) of *H.pylori* (by IMM) and non-*H.pylori* (by MG) in patients with small or large cell primary gastric lymphoma

	Small cell (n=17) pos/n (%)	Large cell (n=35) pos/n (%)
Hp antrum mucosa	7/14 (50.0%)	13/30 (43.3%)
Hp corpus mucosa	12/16 (75.0%)	15/27 (55.0%)
nHp antrum mucosa	6/14 (42.9%)	18/30 (60.0%)
nHp antrum tumour ¹	3/12 (25.0%)	16/24 (66.7%)
nHp corpus mucosa	7/16 (43.8%)	14/27 (51.9%)
nHp corpus tumour ²	4/14 (28.6%)	14/21 (66.7%)

¹ Chi-Square, $p=0.02$; ² Chi-Square, $p=0.03$

The prevalence of *H.pylori* was similar in antrum and corpus mucosa of patients with small cell versus patients with large cell lymphomas (Table VI.4). The density of *H.pylori* was significantly higher ($p=0.009$) in corpus mucosa of patients with a large cell compared with those with a small cell lymphoma (Figure VI.3).

Regarding the non-*H.pylori* flora, the prevalence was higher in tumour of antrum or corpus in patients with a large compared to those with a small cell lymphoma (Table VI.4) (antrum, $p=0.02$; corpus, $p=0.03$). Further differences in the prevalence or density of non-*H.pylori* were not found.

Figure VI.3: Density of *H. pylori* and non-*H. pylori* flora in antrum and corpus of patients with a small cell (A) or large cell primary gastric lymphoma (B) (Percentage of grade 1,2,3 of total number of positives)

Discussion

Using a joint evaluation with the non-specific MG stain for the total intragastric bacterial flora and a specific immunohistochemical stain for the detection of *H.pylori*, non-*H.pylori* flora was detected in 67.3% and *H.pylori* in 61.5% of patients with primary gastric lymphoma. Both types of bacterial flora were simultaneously present in 36.5% of patients. *H.pylori* was only found in non-tumorous gastric mucosa, whereas non-*H.pylori* flora was present in antrum and corpus mucosa as well as on and within the surface of the lymphomas.

In this study using a specific immunohistochemical stain, fewer patients with primary gastric lymphoma were found to be *H.pylori*-positive compared to other studies^{1,3,18}. An explanation for this discrepancy could be that the use of MG and HE has overestimated the *H.pylori* frequency in comparison with the *H.pylori* specific IMM stain as used in this study. Secondly, it cannot be excluded that real differences in the prevalence of *H.pylori* exist between different geographical regions as described by Doglioni et al.¹⁹. Furthermore, it is possible that *H.pylori*-negative patients at present have previously been positive, as it has been described in patients with lymphocytic gastritis^{20,21}. Earlier *H.pylori*-positivity can theoretically be controlled for by serology. Unfortunately, it was not possible to assess seropositivity for *H.pylori* in this study as we performed a retrospective study on histological material only derived from ten different hospitals.

As the archival material of this study was obtained before 1992, *H.pylori* eradication therapy was unlikely to be generally applied. Eradication is therefore not the cause of the low *H.pylori* prevalence found in this study. Relatively low *H.pylori* prevalences in patients with primary gastric lymphoma have recently also been described by others^{2,4}. The prevalence of intragastric *H.pylori*-positivity in this study is not much different from that found in patients with non-ulcer dyspepsia or in healthy blood donors of the same geographical region of a similar age, where *H.pylori* was present in 49.6% and 58.3%, respectively, using serology²². This has to be taken into account for the discussion of the aetiological role of *H.pylori* in gastric lymphoma.

To our knowledge, this is the first study to focus on the presence of non-*H.pylori* flora besides *H.pylori* in primary gastric lymphoma. Non-*H.pylori* flora occurred in a surprisingly high proportion of patients with a primary gastric lymphoma. Several explanations for this phenomenon can be put forward:

- Colonization with non-*H.pylori* flora can be a consequence of a decreased gastric acid secretion due to atrophic gastritis. Mucosal atrophy of the gastric body has been described in lymphocytic gastritis which is common in patients with primary gastric lymphoma^{21,23}.

- The tumour itself can cause decreased acid secretion by involvement of acid secreting gastric mucosa, and altered gastric motility as a consequence of tumour infiltration can lead to a stagnant loop syndrome with bacterial overgrowth. Theoretically, the non-*H.pylori* flora found in non-tumorous gastric mucosa could be spreading out from the tumour area.
- Finally, patients with primary gastric lymphoma might have gastrointestinal complaints and may have been treated with gastric acid inhibitors, resulting in an increased intragastric pH and bacterial overgrowth^{24,25}.

Hitherto, little attention has been paid to the distribution of *H.pylori* and other bacteria in the stomach of gastric lymphoma patients. In this patient group the prevalence of *H.pylori* was somewhat higher in corpus than in antral type mucosa (although not significant). A similar situation is found during acid inhibitory treatment for *H.pylori* associated peptic disorders, where a displacement of *H.pylori* from antrum towards corpus can occur^{26,27}. The finding of a trend of a greater number of *H.pylori* positive corpus- than antrum-specimens in patients with primary gastric lymphomas can support the presence of a reduced intragastric acidity in the present study population. The *H.pylori* density in corpus mucosa of patients with large cell lymphomas is significantly higher than in patients with small cell lymphomas. This may indicate that there can be a more prolonged or stronger reduction in gastric acidity in the former group of patients. In addition, this patient group is significantly older than those with a small cell lymphoma and therefore a longer duration of the *H.pylori* infection with subsequent atrophy may exist.

For non-*H.pylori* bacteria no difference in distribution was found between antrum and corpus or between tumour tissue and gastric mucosa analysing all patients. This homogeneous distribution of non-*H.pylori* flora in the stomach supports the presence of hypochlorhydria or achlorhydria in the stomach as an explanation for the presence of these microorganisms. However, a significantly higher prevalence of non-*H.pylori* was found in tumour tissue of patients with a large cell compared with small cell lymphoma.

H.pylori and non-*H.pylori* bacterial flora were both found with a similar frequency, density and distribution. No differences were found in the subgroups of patients with *H.pylori* only, non-*H.pylori* only, and the joint occurrence of *H.pylori* and non-*H.pylori*. This suggests that there is no direct relation between the two types of flora and that they may be independent from each other.

The role of the non-*H.pylori* bacteria in relation to primary gastric lymphoma is not clear yet. To our knowledge only the study of Blackburn et al.²⁸ has some relation to this subject; in that study an increased mortality from both gastric cancer and leukaemia was found in patients with pernicious anaemia, a condition

known to be associated with intragastric bacterial overgrowth^{29,30}.

On the one hand, the non-*H.pylori* flora could be a consequence of the functional situation in gastric lymphoma patients, resulting from the stagnant loop situation and/or reduced gastric acidity. On the other hand, this flora may also play a role in the development of the lymphomas by being an antigenic stimulus for an inflammatory response. In parallel to gastric cancer, this bacteria could also be responsible for the production of toxic or carcinogenic metabolites, such as N-nitroso-compounds¹¹. The higher prevalence of non-*H.pylori* flora in tumour tissue of large cell compared with small cell lymphomas may be a consequence of differences in tumour mass and/or gastric acidity between both groups as well as the higher age of the former group of patients. However, it cannot be excluded that the non-*H.pylori* flora contributes to the development of a large cell lymphoma. Further studies on the role of the non-*H.pylori* flora in the development and progression of small and large cell primary gastric lymphoma are therefore required.

Independent of the possible pathogenetic role of the non-*H.pylori* flora found in gastric lymphoma, the high frequency and concentrations of this flora in patients with primary gastric lymphoma may interfere with the detection of *H.pylori* and possibly lead to overestimation of *H.pylori*¹². We therefore recommend the use of a *H.pylori*-specific immunohistochemical staining for *H.pylori* for further studies on the role of this bacterium in the pathogenesis and therapy of gastric MALT-lymphomas.

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Chapter VII

The role of *Helicobacter pylori* and non-*Helicobacter pylori* intra-gastric bacterial flora in gastric cancer: a study in 39 newly diagnosed patients

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Summary

Helicobacter pylori (*H.pylori*) is associated with gastric cancer as it may initiate a cascade towards gastric cancer. In achlorhydric atrophic gastritis, *H.pylori* is rarely found, but non-*H.pylori* bacterial flora is frequently occurring.

In 39 consecutive patients with gastric cancer, antral and corpus biopsies were obtained for the histological classification of *H.pylori* by immunohistochemistry (IMM) and non-*H.pylori* bacterial flora by the modified Giemsa (MG) (according to form, location and negative IMM). In 21 patients, biopsies and/or gastric juice were also taken for culture of non-*H.pylori* flora. Current and previous *H.pylori* infection was screened for by IgG and IgA Elisa, and Western blotting. In addition, fasting serum gastrin levels were determined using radioimmunoassays.

H.pylori was detected by IMM in 21 (53.8%), by IgG in 22 (56.4%), by IgA in 13 (33.3%), and by Western blotting in 29 (74.4%) of 39 patients. All IgG, IgA and IMM positive patients were also Western blotting positive. Eight patients (20.5%) were serologically *H.pylori* positive without current histological *H.pylori* infection and ten patients (25.6%) were negative by both histology and serology. Non-*H.pylori* flora was detected by MG in 27 of 39 (69.2%) and by culture in 14 of 21 (66.7%) patients. Non-*H.pylori* flora occurred with a similar frequency in Western blotting positive as in Western blotting negative patients. Elevated gastrin levels and antrum-sparing atrophic gastritis were more frequent in *H.pylori* negative than in *H.pylori* positive patients, indicating a possible role of atrophic gastritis of type A.

In about 25% of gastric cancer patients no prior or current *H.pylori* infection could be demonstrated. In another 20% only serological evidence of *H.pylori* was found, indicating a previous infection. Non-*H.pylori* was found in two-thirds of all patients regardless of the *H.pylori* status. The non-*H.pylori* flora may result from reduced intra-gastric acidity, partly due to type A atrophic gastritis and should be considered as a factor contributing to the development of gastric cancer, possibly via the formation of N-nitroso compounds.

Introduction

Presently there is little doubt about the association between *Helicobacter pylori* (*H.pylori*) infection and the risk of gastric cancer. In serological studies, the odds ratio of a *H.pylori* positive patient to develop gastric cancer appears to be 2.6 to 6.0 that of a *H.pylori* negative person¹⁻⁵. An even higher odds ratio of 13.3 was found for gastric cancer patients under the age of 40 years⁶. *H.pylori* is associated with chronic antral gastritis, which on long-term may progress towards chronic atrophic gastritis and intestinal metaplasia^{7,8}. Both of the latter conditions are related to an increased risk of gastric cancer, especially of the intestinal type^{7,9-11}. However, mucosal atrophy and intestinal metaplasia are inversely related to an actual presence of *H.pylori* in the gastric mucosa^{7,12,13}; this means that *H.pylori* may initiate the pathogenetic sequence towards gastric cancer but must not necessarily play a role throughout the entire process.

Only a small proportion of *H.pylori*-positive subjects will develop gastric cancer¹⁴. Therefore other factors - for example genetic and environmental - will also play a role in the aetio-pathogenesis of gastric cancer. A low vitamin C intake¹⁵⁻¹⁷, a high salt intake^{16,18,19} and food rich in nitrates and nitrites²⁰⁻²³ are associated with an increased risk of gastric cancer. Also high serum gastrin levels may play a role in the gastric carcinogenesis via induction of ECL-cell hyperplasia and subsequent carcinoid formation^{24,25}.

Hypergastrinaemia is found in antrum-sparing atrophic gastritis with or without pernicious anaemia, so-called "type A" gastritis. High serum gastrin levels have been used to screen for atrophic type A gastritis²⁶. Mucosal atrophy in both antrum and corpus gastritis A and B will be characterized by a low or normal serum gastrin²⁷.

We have recently shown that non-*H.pylori* bacterial flora is present in the stomach of patients with primary gastric lymphoma in a high frequency and concentration²⁸. Intra-gastric bacterial overgrowth is also found in clinical situations which may precede the development of gastric cancer, such as atrophic gastritis and pernicious anaemia^{29,30}. Intra-gastric bacteria may contribute to gastric carcinogenesis by formation of potentially carcinogenic N-nitroso compounds from nitrates and nitrites^{29,30,32}.

This was the reason to investigate the simultaneously the past and current presence of *H.pylori* and the current presence of non-*H.pylori* intra-gastric bacterial flora in a consecutive series of newly diagnosed gastric cancer patients by histology, microbiology, and serology. In addition, *H.pylori* positive and *H.pylori* negative patients were compared according to serum gastrin levels and the presence of antrum-sparing atrophic gastritis.

Material and methods

Patients

From January 1992 to December 1994, in a prospective study, all patients suspected to have gastric cancer underwent upper gastrointestinal endoscopy. Of the 82 patients with a confirmed diagnosis of gastric adenocarcinoma, 39 had a complete set of serum samples and gastric biopsy specimens and were included in this study.

During endoscopy fasting intragastric pH of the patients was measured using pH-paper strips with grading steps of 0.5 from pH 0 to pH 14 (Schleicher & Schüll GmbH; Dassel, Germany).

Histological assessment of the intragastric bacterial flora

Antrum and corpus mucosal biopsies were obtained during endoscopy, fixed in formalin and embedded in paraffin. Serial sections were cut for Haematoxylin-Eosin stain (HE), modified Giemsa stain (MG)³³ and immunohistochemistry (IMM)³⁴. Adenocarcinoma were classified as intestinal or diffuse type on HE stained sections³⁵ and non-tumorous antrum and corpus biopsies were scored for the presence of mucosal atrophy according to the Sydney classification³⁶.

For the detection and histological classification of the intragastric bacteria, the IMM and MG were used. IMM with a purified polyclonal antiserum (DAKO B471, ITK diagnostics BV, Uithoorn, The Netherlands) was used to detect *H.pylori*. MG was used to screen for the presence of bacteria, and for preliminary differentiation between *H.pylori* and non-*H.pylori* flora on the basis of morphology and location. In MG positive patients, presence of non-*H.pylori* bacteria were assumed when an IMM stain of an adjacent section was *H.pylori*-negative on that position.

Microbiological assessment of the intragastric bacterial flora

From a randomly selected subgroup of patients (n=21) biopsies of antrum and/or corpus and gastric juice were also cultured. Biopsies were washed thrice in sterile saline to remove contaminating flora³⁷ and transferred to agar plates with a sterile forceps. Blood agar plates to assess total aerobic bacterial growth were inoculated using the five segments method³⁸. Oropharyngeal flora and Enterobacteriaceae were identified using standard microbiological methods³⁹. Oropharyngeal flora included *Neisseria* spp., *Streptococci* spp., and *Corynebacterium* spp.

***Helicobacter pylori* serology**

Shortly after endoscopy, blood samples were taken after an overnight fast. The samples were centrifuged and the sera were stored immediately at -20°C until further analysis. IgG and IgA antibodies against *H.pylori* were detected with commercial Elisa assays (Pyloriset EIA-G, Pyloriset EIA-A) (Imphos BV, Zambon group, Amersfoort, The Netherlands). The assays used an extracted whole antigen preparation. All 39 samples were tested within one assay. The cut off values for positivity were used according to the manufacturers' instructions. Patients with a serum titer ≥ 300 units for IgG and ≥ 250 units for IgA were considered to be *H.pylori* positive.

Furthermore, a commercial Western blotting for IgG (Helicoblot 2.0, Imphos BV, Zambon Group, Amersfoort, The Netherlands) was performed in duplo with heat-inactivated serum samples. Bands were present for 19.5, 26.5, 30, 35, 89 (vacA), and 116 kD (cagA) proteins. When two bands of the 19.5, 26.5, or 30 kD proteins, or any of the bands among the 35, 89, or 116 kD proteins were positive, the patient was considered to be *H.pylori* positive (as indicated by the manufacturer). *H.pylori* positive and negative control sera were included each time the test was performed.

Serum gastrin

Serum gastrin was determined using a radioimmunoassay⁴⁰. The reference value for normality was < 85 pg/ml.

Statistical analyses

For the purpose of this study, the patient was considered to either be or to have been *H.pylori*-infected when one or several of the *H.pylori*-specific tests (IMM, IgG, IgA or Western blotting) were positive. Comparison of gender, tumour type and tumour site (i.e. antrum versus corpus), the presence of antrum-sparing atrophic gastritis and the prevalence of non-*H.pylori* bacteria in relation to the prevalence of *H.pylori* were analyzed by a Chi-Square test, if necessary with the Fisher's exact test. The intragastric pH, age, and serum gastrin levels in *H.pylori* positive patients were compared with *H.pylori* negative patients using the Mann-Whitney U test. A p-value less than 0.05 was considered to be significant.

Results

Complete serological and histological data were available for 39 gastric cancer

patients, 19 male and 20 female with a median age of 76 years (range 48 to 93). The fasting intragastric pH was measured at endoscopy in 28 patients. Median pH was 6.3 (range 1.0-8.0). In 21 patients (75.0%), the pH was above 3.5.

The tumour was located in the antrum of 10, in the corpus/cardia of 23, and in both antrum and corpus of 5 patients; one patient had a Billroth II stomach. Thirty patients had an intestinal type gastric adenocarcinoma and nine a diffuse type. In Table VII.1 the distribution of the tumour site according to the tumour type is given. The intestinal type of gastric cancer was more frequently located in the corpus than the diffuse type of gastric cancer ($p < 0.05$).

Histological assessment of intragastric bacteria

At least two biopsy specimens of non-tumorous gastric mucosa were present per anatomical site in 37 patients. From two patients only one non-tumorous corpus biopsy was available.

Using IMM, *H.pylori* was found in antrum and/or corpus of 21 (53.8%) patients. Non-*H.pylori* flora was identified in 27 (69.2%) patients by the combined MG and IMM stains. *H.pylori* only, non-*H.pylori* bacteria only, or both types of flora were found in 7 (17.9%), 13 (33.3%), and 14 (35.9%) patients, respectively. In the remaining five patients (12.8%) no bacteria were detected by histology.

Table VII.1: The occurrence of the tumour sites according to the tumour type (intestinal versus diffuse)

Tumour site	Intestinal type n=30	Diffuse type n=9
	n (%)	n (%)
corpus ¹	21 (70.0%)	2 (22.2%)
antrum	5 (16.7%)	5 (55.5%)
corpus/antrum	3 (10.0%)	2 (22.2%)
BII stomach	1 (3.3%)	- (0.0%)

¹ Intestinal type adenocarcinoma were significantly more frequent in corpus than diffuse type: Chi-Square $p < 0.05$)

Microbiological assessment of the intragastric bacterial flora

In 19 patients, biopsies were present for culture. In another two patients only gastric juice was obtained for culture. Gastric biopsy cultures were positive in 12 of 19 (63.2%) patients: oropharyngeal flora only was found in nine patients; Enterobacteriaceae only were found in two patients (*Klebsiella* spp., *Escherichia*

coli); in one patient both oropharyngeal flora and *Klebsiella* spp. were cultured. Of the two patients with gastric juice only, culture showed oropharyngeal flora in one; in the other patient both oropharyngeal and *Escherichia coli* were found.

In 10 of these 12 biopsy culture positive patients (83.3%), non-*H.pylori* flora was also demonstrated in the MG stain. Two patients were culture positive but MG negative. Another four patients were culture negative, but MG positive for non-*H.pylori* flora.

Helicobacter pylori serology

Using Western blotting, 29 of 39 patients (74.4%) were found to be *H.pylori* positive. In 26 of 29 Western blotting positive patients (89.7%), antibodies against the 116 kD band for *cagA* were present. IgG and IgA antibodies were detected with Elisa assays in 22 patients (56.4%) and 13 patients (33.3%), respectively (Figure VII.1).

Figure VII.1: Presence of *H.pylori* by Western blotting, IgG, IgA, and immunohistochemistry for 39 gastric cancer patients

No IgG-positive or IgA-positive patient were missed by Western blotting. Five patients were only Western blotting positive and negative with the IgG and IgA tests (Figure VII.1). An example of a *H.pylori*-positive and a *H.pylori*-negative Western blotting is shown in Figure VII.2.

No IMM positive patients were missed by Western blotting, but two and nine IMM positive patients were IgG- and IgA-negative, respectively. Six Western blotting positive patients and three IgG positive patients were IMM negative (Figure VII.1).

Figure VII.2: Examples of *H.pylori* positive (no. 1 and 2) and *H.pylori* negative results (no. 3 and 4) by Western blotting

Helicobacter pylori positive versus *Helicobacter pylori* negative patients

In total, 29 patients were *H.pylori* positive by one or several tests (always positive by Western blotting). Ten patients were *H.pylori* negative both by histology and by all serology tests. Comparing Western blotting-positive with Western blotting-negative patients (Table VII.2) no significant differences were found according to age, gender and fasting intragastric pH. However, *H.pylori* was found significantly more often in diffuse than intestinal type carcinoma patients ($p < 0.05$). The presence of non-*H.pylori* flora was as frequently found in *H.pylori*-positive as *H.pylori*-negative patients.

Comparing gastric cancer patients according to the type and site of the tumour, a similar distribution of *H.pylori* and non-*H.pylori* flora was found in all groups. However, the tumour of patients with a *cagA* positive strain was significantly more often located in the antrum than in the corpus ($p < 0.05$). Furthermore, intestinal type patients tended to be older than diffuse ones ($p = 0.06$).

and patients with a tumour in the corpus were older than those with a tumour in the antrum ($p < 0.05$).

In ten *H. pylori*-negative patients, non-*H. pylori* bacteria were found in seven patients (70.0%). Of the eight patients *H. pylori*-positive by serology only, non-*H. pylori* bacteria were present in six patients (75.0%).

Table VII.2: Patient characteristics and presence of non-*H. pylori* bacterial flora in *H. pylori*-positive and *H. pylori*-negative patients (by Western blotting)

	<i>H. pylori</i> positive n = 29		<i>H. pylori</i> negative n = 10	
Age				
median(range)	75	(51-93)	80	(48-90)
Sex				
male/female	14/15		5/5	
pH g. juice				
median(range)	n=20 6.8	(1.5-8.0)	n=8 5.5	(1.0-7.0)
Tumour location				
corpus	14	(48.3%)	9	(90.0%)
antrum	9	(31.0%)	1	(10.0%)
antrum/corpus	5	(17.2%)		
BII	1	(3.4%)		
Tumour type¹				
intestinal	20	(69.0%)	10	(100.0%)
diffuse	9	(31.0%)	0	(0.0%)
MG non-<i>H. pylori</i>	20/29	(69.0%)	7/10	(70.0%)
Culture non-<i>H. pylori</i>	11/16	(68.8%)	3/5	(60.0%)

¹ Higher *H. pylori* prevalence was found in diffuse than intestinal type adenocarcinoma patients: Chi-Square $p < 0.05$

Serum gastrin

The median serum gastrin level of all patients was 116 (12-5580) pg/ml with a gastrin value above the reference value in 25 of 39 (64.1%) patients. Median gastrin values tended to be higher in *H. pylori* negative (n=10) than in *H. pylori* positive (n=29) patients ($p=0.07$) (Figure VII.3). No association of serum gastrin levels was found with the histological prevalence of *H. pylori* (by IMM) or non-*H. pylori* bacterial flora (by MG minus IMM).

Antrum-sparing atrophic gastritis was found in 5 of 10 *H.pylori* negative patients (50.0%) and in 4 of 29 *H.pylori* positive patients (13.3%) ($p < 0.05$). Furthermore, the presence of this type of gastritis corresponded with high gastrin levels in all five *H.pylori* negative patients but only in one of four *H.pylori* positive patients.

Figure VII.3: Median serum gastrin values in *H.pylori* positive (n=29) and *H.pylori* negative (n=10) gastric cancer patients (by Western blotting)

Discussion

In this prospective series of gastric cancer patients, in 25% there was no indication of previous or current *H.pylori* infection, whilst current *H.pylori* infection was found in 53.8% by immunohistochemistry and previous or current infection in 74.4% by serology, including Western blotting. Current infection with non-*H.pylori* intragastric bacterial flora was found histologically in 69.2% of gastric cancer patients to a comparable degree in *H.pylori*-positive and *H.pylori*-negative patients.

The serological *H.pylori* positivity of the patients is in line with those reported by others using either an IgG-Elisa assay or immunoblotting^{1,41-43}. Histologically, higher *H.pylori* prevalences are reported in the literature; especially for the intestinal type of gastric cancer prevalences of 88-89% were found^{44,45}, and even 94% using a combination of culture and histology⁴⁶. However, also prevalences around 60% are described^{13,47}.

In most studies, HE and MG, both non-specific staining methods, were applied for the histological detection of *H.pylori*. Use of a specific immunohistochemical stain for the detection of *H.pylori* may explain the lower prevalences found in this study. In addition, special attention was paid to the presence of non-*H.pylori* bacteria when using the MG, thereby excluding some falsely *H.pylori*-positive patients.

Serological tests are of great value in screening for *H.pylori*-positivity^{48,49}. Both IgA and IgG Elisa assays used in this study^{50,51} appeared to be highly reproducible for the assessment of *H.pylori*-positivity or -negativity.

In this group of gastric cancer patients, Western blotting for the detection of IgG antibodies against *H.pylori* seemed to be the most sensitive method. No *H.pylori* positives diagnosed by IMM, IgG or IgA Elisa were missed by Western blotting. However, compared to IMM about 20% additional *H.pylori* positive patients were detected by Western blotting. The presence of *H.pylori* antibodies in this group of patients probably indicates a past *H.pylori* infection.

Using both serology and histology, 25% of the gastric cancer patients were negative for a past or present *H.pylori* infection. Although it cannot be excluded that disappearance of *H.pylori* on long term has resulted in a decrease or even disappearance of the antibody titers⁵², similar prevalences of *H.pylori* negative gastric cancer patient were found by Sipponen et al.⁵³ also. We believe that one fourth of the patients has developed gastric cancer without a prior *H.pylori* infection and that other aetio-pathogenetic factors have to be considered in these.

Comparing *H.pylori* positive with *H.pylori* negative patients (taking Western blotting as discriminator), no significant differences were seen concerning age, gender, intragastric pH, or tumour site. However, diffuse type adenocarcinoma were more frequently *H.pylori* positive than intestinal type carcinoma. This finding does therefore not support the hypothesis, that *H.pylori* is mainly involved in the pathogenesis of the intestinal type of cancer, a concept that has also been challenged by others^{43,45,53}.

About 90% of *H.pylori* positive patients had antibodies against cagA, supporting the hypothesis that cagA positive strains may be associated with an increased risk of gastric cancer⁵⁴. However, no differences were found between the prevalences of cagA in patients with an intestinal or diffuse type of gastric cancer.

In severe atrophic gastritis, a reduced or absent intragastric acidity is frequently found and is associated with intragastric bacterial overgrowth^{29,30,55-57} and an increased risk of gastric cancer⁵⁸⁻⁶⁰. In the gastric cancer patients of this study, non-*H.pylori* bacterial flora was found frequently and in high amounts in gastric biopsy specimens by MG as well as by culture. This seems to be a consequence of low gastric acid secretion. Unfortunately, stimulated acid secretion could not be

determined in this patient population.

The intragastric concentration of non-*H.pylori* flora are not assumed to result from contamination only, as culture of biopsy specimens has confirmed the histological findings in most patients. Before culture all biopsies had been washed thrice in order to remove contaminating flora. Furthermore the non-*H.pylori* bacterial flora was found not only on the surface of the gastric mucosa but also within the gastric glands.

Two-thirds of the patients positive by histology or serology for *H.pylori*, were in addition positive for non-*H.pylori* bacterial flora. Also in 70% of *H.pylori* negative patients, non-*H.pylori* flora was found. This could mean that the presence of non-*H.pylori* flora in gastric cancer patients is independent of previous or current *H.pylori* infection. It also suggests that growth of non-*H.pylori* flora is not only related to atrophic gastritis induced by *H.pylori*, but may also result from atrophic gastritis of other aetiology such as autoimmune.

The fact that eight patients with serological evidence of *H.pylori* infection, had no current infection but only evidence of non-*H.pylori* intragastric bacteria could support the hypothesis that *H.pylori* associated gastritis develops towards gastric atrophy with hypochlorhydria or achlorhydria, subsequent bacterial overgrowth and production of N-nitroso compounds^{29,30,32}. The N-nitroso compounds may then play a role in the final development of gastric cancer. Another explanation for the association between intragastric bacterial overgrowth is a low intragastric vitamin C concentration^{16,17}. Ascorbic acid acts as a scavenger of reactive oxygen and inhibits the N-nitrosation reaction¹⁵. Partial deficiency of vitamin C as found in *H.pylori* positive gastritis^{61,62}, and in situations with reduced intragastric acidity and bacterial overgrowth^{17,63}. Furthermore, it can be hypothesized that non-*H.pylori* intragastric bacteria may contribute to the development of gastric cancer by acting as an antigenic trigger.

The elevated levels of serum gastrin with the presence of antrum-sparing atrophic gastritis found in this group of gastric cancer patients were predominantly present in the *H.pylori* negative patients. This finding might indicate that a type A atrophic gastritis is associated with gastric carcinogenesis in *H.pylori* negative patients.

In summary, in 25% of a consecutive series of gastric cancer patients, no evidence was found for current or previous *H.pylori* infection. Non-*H.pylori* bacterial flora was found in about two-thirds of all patients and has to be considered as a factor that may contribute to the development of gastric cancer. All diffuse cancers were *H.pylori* positive but only 67% of the intestinal type. Antrum-sparing type A atrophic gastritis with elevated serum gastrin seems to be frequent in *H.pylori* negative patients with gastric cancer.

It can be concluded that other factors than *H.pylori* may contribute to the aetio-pathogenesis of gastric cancer and that non-*H.pylori* flora may be one of those.

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Chapter VIII

General discussion

Introduction

In this thesis, the contribution of both *H.pylori* and non-*H.pylori* intragastric bacterial flora in patients with peptic ulcer disease, primary gastric lymphoma and gastric cancer has been studied. Therefore, culture procedures were optimized and a specific immunohistochemical stain for the histological detection of *H.pylori* was applied in combination with the non-specific modified Giemsa stain to assess the total intragastric bacterial flora.

Detection of *Helicobacter pylori* by culture

The sensitivity of culture for the detection of *H.pylori* is often found to be moderate and is influenced by low counts of *H.pylori*, transport time and conditions and the presence of contaminating flora. In the literature, use of several selective media containing different combinations of antibiotics have been described to detect *H.pylori* in the presence of non-*H.pylori* flora. We further optimized culture procedures to improve the detection of *H.pylori* by washing and grinding gastric biopsy specimens before inoculation, in addition to the use of selective media (Chapter II). Washing of the specimens reduced the amount of contaminating flora but not of *H.pylori* and thereby improved the detection of the latter. Also grinding of the specimens, as applied in this study, results in a higher bacterial yield of *H.pylori*. Furthermore, biopsies were kept at 4°C and cultured within four hours to prevent a decreased viability of the *H.pylori* microorganisms.

Detection of *Helicobacter pylori* by histology

For the histological detection of *H.pylori*, mostly non-specific staining methods are used with a moderate agreement between observers¹. Especially in situations with a high intragastric pH and consequently bacterial overgrowth in the stomach, a sensitive and specific staining method for the histological detection of *H.pylori* is required. A specific immunohistochemical stain employing a commercially available antiserum for the detection of *H.pylori* was evaluated in situations where *H.pylori* and/or non-*H.pylori* bacterial flora could be present (Chapter III and IV). Both a high sensitivity and specificity and the lowest inter-observer variation were found for the immunohistochemical stain compared to the modified Giemsa stain (lowest specificity) and the Warthin-Starry silver stain (lowest sensitivity).

The presence of non-*H.pylori* bacteria will result in false positive and false negative results, and a large inter-observer variation of *H.pylori* scores in the MG stain. This stain has to diagnose *H.pylori* on the basis of morphology and location only. These problems can be overcome by use of a specific immunohistochemical

stain for the histological detection of *H.pylori*. Furthermore, coccoid forms of *H.pylori* can be missed using non-specific staining methods, but react positively in the immunohistochemical stain.

Also PCR will be a sensitive and specific method to detect *H.pylori* in the presence of other bacteria. *H.pylori* can also be detected by non-invasive tests such as the urea breath test². However, the sensitivity and specificity of this test can be influenced by treatment with antibiotics, gastric acid inhibitors, or other clinical conditions associated with a reduced gastric acid secretion.

When it is desirable to distinguish symptomatic from asymptomatic *H.pylori* infection, endoscopy is required³. Many studies still use culture, histology or a combination of both for the detection of *H.pylori*. Megraud⁴ concludes in a review on *H.pylori* diagnosis that both histology and culture give very good results when performed under optimal conditions, in addition to information about the status of the mucosa. The present study has contributed to the optimisation of both culture and histology for the detection of *H.pylori*.

Prevalence of *Helicobacter pylori* and non-*Helicobacter pylori* flora in peptic ulcer disease, primary gastric lymphoma and gastric cancer patients

In the gastric disorders studied in this thesis, the prevalence of *H.pylori* was lower than found in the literature (Table). This may be due to the use of the specific immunohistochemical stain for the detection of *H.pylori* in our studies. Especially when other flora is present, use of the modified Giemsa or the Haematoxylin-Eosin stain may result in false positive scores. In all studies of this thesis, special attention was paid to the presence of non-*H.pylori* flora in the modified Giemsa stain. However, in mainly untreated DU and GU patients, hardly any non-*H.pylori* flora could be detected by the modified Giemsa and there was a good correlation between the modified Giemsa and the immunohistochemical stain. In patients with treated acid-related disorders, with gastric cancer, and with primary gastric lymphoma, high amounts of non-*H.pylori* were found. This can disturb the detection of *H.pylori* and will warrant the use of a specific immunohistochemical staining method for the histological detection of *H.pylori*.

Prevalence of *H.pylori* (by immunohistochemical stain) and non-*H.pylori* flora (by modified Giemsa) in patients with peptic ulcer disease, primary gastric lymphoma, and gastric cancer

Clinical condition	<i>H.pylori</i>	non- <i>H.pylori</i>
Duodenal ulcer	81.6%	10.2%
Gastric ulcer	83.0%	17.0%
Gastric lymphoma	61.5%	67.3%
Gastric cancer	53.8%	69.2%

Duodenal versus gastric ulcer patients

DU and GU patients were further compared to assess differences in the gastroduodenal distribution, prevalence and density of *H.pylori* as well as the histological changes which may contribute to a differential presentation of both forms of peptic ulcer disease. The *H.pylori* prevalence was found to be similar in antrum and corpus of both DU and GU patients. The *H.pylori* density was lower in corpus than antrum of DU and was higher in corpus of GU than of DU patients. Together with the antral-predominant gastritis in DU and the more wide-spread inflammatory changes in GU patients, these findings were in line with reports in the literature⁵⁻⁷.

Higher prevalences of the precancerous lesions atrophic gastritis and intestinal metaplasia in GU than DU patients support the hypothesis that GU patients are at higher risk to develop gastric cancer than DU patients⁸⁻¹⁰. From the presented study in this thesis it is not clear whether the histological differences between DU and GU patients result from or are the cause of the differences in *H.pylori* colonization.

One factor reported to be specifically associated with *H.pylori* and duodenal ulcer disease, is the presence of gastric metaplasia in the duodenal bulb. In contrast with findings in the literature^{11,12}, we could not confirm a role of gastric metaplasia in the development of a duodenal ulcer as similarly low prevalences were found in both DU and GU patients. An alternative ulcerogenic mechanism may be the release of inflammatory mediators in the gastric lumen that subsequently enter the duodenum. However, a comparable prevalence of *H.pylori* and gastritis in antrum of DU and GU patients does not either support a role of this mechanism in the differential pathogenesis of DU and GU formation.

Several reviews ascribe a role to the altered gastric acid secretion and the age of acquisition of *H.pylori* infection in the differential pathogenesis of peptic ulcer disease: Acquisition of *H.pylori* infection early in life should increase the risk of developing gastric ulcer but not duodenal ulcer⁸, and DU patients have an increased

acid secretion while GU patients are characterized by a normal or decreased acid secretion^{7,9,13}. In this context, an increased parietal cell mass, increased basal acid secretion, rapid gastric emptying and an increased duodenal acid load may predispose to DU formation¹⁴. A higher basal intragastric pH in GU than in DU patients was confirmed in our study.

Gastric malignancies

Non-*H.pylori* flora was found in a relatively high proportion of both patients with primary gastric lymphoma (Chapter VI) and patients with gastric cancer (Chapter VII), with a similar degree in *H.pylori* positive and *H.pylori* negative patients. Although the non-*H.pylori* flora might just be a consequence of the functional situation in both patient groups, it cannot be excluded that this flora significantly contributes to their pathogenesis: by being an antigenic stimulus for a chronic inflammatory response or by the production of toxic and/or carcinogenic metabolites, such as N-nitroso compounds. Finally, both *H.pylori* and non-*H.pylori* flora are associated with a low intragastric vitamin C level^{15,16}. Vitamin C can protect against gastric cancer by preventing the formation of potentially carcinogenic N-nitroso compounds and reactive oxygen metabolites. The known presence of non-*H.pylori* flora in gastric cancer precursor lesions or conditions supports a possible role of this flora in gastric carcinogenesis¹⁷.

Patients in whom no *H.pylori* is present in the stomach at the time of diagnosis, can previously have been infected by *H.pylori* without signs of a current infection. This can be controlled for by serology. Unfortunately, serum samples were not available for the patients with primary gastric lymphoma, but they were for the gastric cancer patients. In these patients, *H.pylori* was detected in 53.8% by immunohistochemistry and in 74.4% by serology (i.e. Western blotting). Using the same Western blotting technique, *H.pylori* was detected in 62.5% of healthy blood donors of the same age and the same area. One quarter of gastric cancer patients showed no evidence of a previous or current *H.pylori* infection and about 20% was positive by serology only. The latter indicates that *H.pylori* may in many patients play an initial role in gastric carcinogenesis as suggested by Correa¹⁸: *H.pylori* associated gastritis can progress towards atrophic gastritis and intestinal metaplasia, both precancerous lesions for the intestinal type of gastric cancer. *H.pylori* colonization decreases and potentially disappears with increasing atrophic gastritis and intestinal metaplasia. *H.pylori* associated gastritis can also be the base for the diffuse type of gastric cancer by formation of hyperplasia through involvement of genes affecting cell-cell and cell-matrix junctional proteins¹⁹.

In this study about 90% of *H.pylori* positive gastric cancer patients were cagA positive. The presence of cagA positive *H.pylori* isolates may be associated

with an increased risk of gastric cancer²⁰. This is in line with findings of Kuipers et al.²¹, who reports an increased risk for patients infected with *cagA* positive strains to develop atrophic gastritis.

Although many studies describe an association of *H.pylori* with gastric cancer, this association is not always found to be as strong as reviewed by Crespi et al.²². In our study, the *H.pylori* prevalences both in patients with gastric cancer and with primary gastric lymphoma are not much different from that of the normal population of a similar age and at least in a quarter of the gastric cancer patients. Other factors than *H.pylori* have to be involved in the aetio-pathogenesis of gastric cancer. One of these factors may be the presence of type A autoimmune atrophic gastritis²³: higher prevalence of antrum-sparing atrophic gastritis and higher serum gastric levels were found in *H.pylori* negative than *H.pylori* positive gastric cancer patients.

General conclusions and recommendations

The presence of gastric non-*H.pylori* flora, as found in patients with low acid secretions and gastric malignancies, will warrant the use of specific and sensitive methods for the detection of *H.pylori*, like a specific immunohistochemical stain, optimized culture procedures, or the use of PCR.

The rather small differences in distribution of a *H.pylori* infection and of the frequency of gastric metaplasia between DU and GU patients indicate that *H.pylori* and gastric metaplasia of the duodenum are not the only factors responsible for the differential development of peptic ulcer disease. Other factors have to be studied in addition, including host factors, inflammatory mediators, *H.pylori* strains differences and environmental factors.

H.pylori prevalences found in patients with primary gastric lymphoma and gastric cancer are not much different from the normal population. Furthermore, high amounts of non-*H.pylori* flora have been found in both patients with primary gastric lymphoma and gastric cancer. It cannot be excluded that this flora plays a role in the initiation, development or progression of these gastric malignancies. The exact role of this flora has to be further investigated.

The Western blotting is the most sensitive serological detection method to assess a current or previous *H.pylori* infection and can give additional information on the presence of *cagA* positive strains. This is important as these strains seem to be

related to the development of peptic ulcer disease, atrophic gastritis, and gastric cancer. Their role in primary gastric lymphoma needs further study.

In further studies the non-*H.pylori* bacterial flora of the stomach has to be further characterized to obtain more information on its potentially harmful effects. This can be done by culture of both aerobes and anaerobes and assessment of the presence of nitrate-reducing bacteria. Other options may be the use of PCR or immunohistochemistry to identify single bacterial species or a subgroup of bacteria.

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Chapter IX

Summary

Summary

The presence of a *Helicobacter pylori* (*H.pylori*) infection is associated with duodenal and gastric ulcer disease, gastric cancer and primary gastric (mucosa-associated lymphoid tissue) lymphoma. If the intragastric pH increases, also non-*H.pylori* bacteria can colonize the stomach. The presence of non-*H.pylori* flora may disturb the detection of *H.pylori* both by culture and histology. In this thesis some methodological studies are described on an improved detection of *H.pylori* by culture and immunohistochemistry, especially when *H.pylori* and/or non-*H.pylori* bacterial flora can be present. These techniques are then applied in clinical situations associated with both types of flora.

Regarding the methodological findings of this study, the use of selective agar plates in addition to washing and grinding of the gastric biopsies prior to inoculation, improved the detection of *H.pylori* by culture. The rate of recovery of low amounts of *H.pylori* ($\leq 10^3$ cfu/ml) was low in the presence of high amounts of oropharyngeal flora ($\geq 10^4$ cfu/ml). Pretreatment of the specimens by washing of the biopsies improved the detection of *H.pylori* in 10 of 28 patients.

An immunohistochemical staining method using a purified polyclonal antiserum had a high sensitivity (83.8%) and the highest specificity (90.0%) for the detection of *H.pylori* compared with the modified Giemsa (90.0% and 53.8%, respectively) and the Warthin-Starry silver stain (70.0% and 82.5%, respectively). A non-*H.pylori* flora could for example be found after eradication therapy when patients were treated with gastric acid inhibitors until control endoscopy and can lead to both false positive and false negative results with a high inter-observer variation when non-specific histological staining methods are used.

Using an immunohistochemical stain, the *H.pylori* prevalences were 81.6% in duodenal ulcer, 83.0% in gastric ulcer, 61.5% in primary gastric lymphoma and 53.8% in gastric cancer patients. In all patients, special attention was also paid to the presence of non-*H.pylori* flora through assessment of form and location of microorganisms in the modified Giemsa stain in combination with absent immunoreactivity for *H.pylori* in the same area of a consecutive section. The distribution and density of both floras were studied and related to histological findings.

Non-*H.pylori* bacteria were found in 10.2% of duodenal ulcer and 17.0% of gastric ulcer patients. The immunohistochemical stain and the modified Giemsa stain were comparable for the detection of *H.pylori*: 98.0% concordance in duodenal ulcer and 89.4% concordance in gastric ulcer patients. *H.pylori* prevalences were similar in

corpus, antrum and duodenum of both duodenal and gastric ulcer patients. Only the *H.pylori* density was higher in antrum than corpus of duodenal ulcer and in the corpus of gastric ulcer than of duodenal ulcer patients. The *H.pylori* density was in line with the presence of an antrum-predominant gastritis in duodenal ulcer patients and a more wide-spread gastric mucosal inflammation in gastric ulcer patients. A major role of gastric metaplasia of the duodenal bulb in the differential pathogenesis of duodenal and gastric ulcer could not be confirmed. Higher prevalences of precancerous lesions such as atrophic gastritis and intestinal metaplasia were found in gastric than duodenal ulcer patients, possibly associated with an increased risk to develop gastric cancer.

In patients with primary gastric lymphoma and gastric cancer, a similar frequency of *H.pylori* (61.5% and 53.8%, respectively) and non-*H.pylori* flora (65.4% and 69.2%, respectively) was found. The *H.pylori* prevalences found by immunohistochemistry were lower compared to findings reported in the literature, and were not much different from the normal population of a similar age.

In patients with primary gastric lymphoma, the *H.pylori* prevalences tended to be higher in corpus (62.8%) than antrum mucosa (45.5%), with a higher density in corpus of large than of small cell lymphoma patients. Also in tumour tissue, the prevalence of non-*H.pylori* flora was higher in large than small cell lymphoma. Both observations can be a consequence of differences in tumour mass and/or intragastric acidity.

In gastric cancer patients no significant differences in the presence of *H.pylori* or non-*H.pylori* flora were found in relation to tumour type or tumour site. In these patients, a previous *H.pylori* infection was controlled for by serology: 20% were *H.pylori* positive by serology but not by histology supporting the hypothesis of an initial role of *H.pylori* in the gastric carcinogenesis. However, in a quarter of the gastric cancer patients *H.pylori* could not at all be detected either by serology or histology. In this group of patients other factors than *H.pylori* have to play a role in the pathogenesis of gastric cancer such as type A atrophic gastritis. This type of gastritis was found in 50% of *H.pylori* negative gastric cancer patients. Another factor may be the presence of relatively high amounts of non-*H.pylori* bacteria found in both patient groups with primary gastric lymphoma and gastric cancer. The non-*H.pylori* may contribute to the development or progression of gastric malignancies by acting as an antigenic trigger or by the production of carcinogenic or toxic metabolites.

The role of the total bacterial flora, *H.pylori* and non-*H.pylori*, in the pathogenesis of gastric malignancies needs further study and will warrant the use of a specific

immunohistochemical stain for the histological detection of *H.pylori* in combination with highly sensitive serology.

Chapter X

Samenvatting

Samenvatting

De aanwezigheid van *Helicobacter pylori* (*H.pylori*) in de maag is geassocieerd met het voorkomen van ulcus duodeni, ulcus ventriculi, maagkanker en primaire maag-lymfomen. Een veel toegepaste therapie voor *H.pylori* eradicaatie is een combinatie van twee antibiotica met een maagzuurremmend middel. Als de pH in de maag toeneemt, kunnen ook andere bacteriën, de zogenaamde non-*H.pylori* bacteriën, de maag koloniseren. De aanwezigheid van deze non-*H.pylori* flora kan de detectie van de *H.pylori* met behulp van kweek of histologie verstoren. Dit proefschrift beschrijft methodologische studies betreffende een verbeterde detectie van *H.pylori* met behulp van kweek en immunohistochemie, met name in die omstandigheden waarbij *H.pylori* en/of non-*H.pylori* bacteriën aanwezig kunnen zijn. Vervolgens zijn deze technieken toegepast in een aantal klinische situaties die geassocieerd zijn met het voorkomen van beide types flora.

Uit de methodologische studies is gebleken dat het gebruik van selectieve agar platen in combinatie met het wassen van de maagbiopten en deze vervolgens te vernalen vóór het enten, de detectie van *H.pylori* met behulp van kweek verbeteren. Met name het terugvinden van lage concentraties *H.pylori* ($\leq 10^3$ cfu/ml) werd bemoeilijkt in de aanwezigheid van relatief grote hoeveelheden orofaryngeale flora ($\geq 10^4$ cfu/ml). Wassen resulteerde voornamelijk in een afname van orofaryngeale flora en niet *H.pylori* wat leidde tot een verbeterde detectie van *H.pylori* in 10 van de 28 patiënten.

Immunohistochemie met gebruik van een gezuiverd polyklonaal antiserum tegen *H.pylori*, had een hoge sensitiviteit (83.8%) en de hoogste specificiteit (90.0%) voor het aantonen van *H.pylori* in vergelijking met de gemodificeerde Giemsa kleuring (respectievelijk 90.0% en 53.8%) en de Warthin-Starry zilver kleuring (respectievelijk 70.0 en 82.5%). Non-*H.pylori* bacteriën konden onder andere worden aangetroffen na gebruik van maagzuurremmende middelen gericht op eradicaatie van *H.pylori*. Indien niet-specifieke histologische kleuringsmethoden werden gebruikt voor het aantonen van *H.pylori* na maagzuurremmende therapie, kon de non-*H.pylori* flora leiden tot zowel vals-positieve als vals-negatieve resultaten. Ook tussen beoordelaars werd een grote variatie in beoordeling van de preparaten waargenomen bij gebruik van niet-specifieke kleuringen.

Met behulp van immunohistochemie werd *H.pylori* aangetoond in 81.6% van de ulcus duodeni patiënten, in 83.0% van de ulcus ventriculi patiënten, 61.5% van de patiënten met een primair maaglymfoom en 53.8% van de patiënten met maagkanker. Tevens werd gekeken naar de aanwezigheid van non-*H.pylori*

bacteriën met behulp van de gemodificeerde Giemsa kleuring op basis van vorm en ligging van microörganismen én een negatieve uitslag van de immunohistochemische kleuring voor *H.pylori* op dezelfde locatie van een achtereenvolgende coupe. De verdeling en de dichtheid van *H.pylori* en non-*H.pylori* flora werden bestudeerd en gerelateerd aan de histologische bevindingen.

Non-*H.pylori* bacteriën werden slechts in 10.2% van ulcus duodeni en in 17.0% van ulcus ventriculi patiënten waargenomen. Er was een grote mate van overeenstemming wat betreft het aantonen van *H.pylori* tussen de immunohistochemie en de gemodificeerde Giemsa kleuring: 98.0% in ulcus duodeni en 89.4% in ulcus ventriculi patiënten. De prevalentie van *H.pylori* was gelijk in corpus, antrum en duodenum van ulcus duodeni en ulcus ventriculi patiënten. Echter bij ulcus duodeni patiënten was de dichtheid van *H.pylori* hoger in antrum dan in corpus en de dichtheid was hoger in het corpus van ulcus ventriculi patiënten ten opzichte van ulcus duodeni patiënten. De verdeling van de dichtheid van *H.pylori* over de maag kwam overeen met de aanwezigheid van gastritis die overheersend voorkwam in het antrum bij ulcus duodeni patiënten en een meer gelijkmatig verspreid aanwezig was in antrum en corpus bij ulcus ventriculi patiënten. In deze studie kon niet worden bevestigd dat de aanwezigheid van gastrische metaplasie in de bulbus duodeni een belangrijke rol speelt in de differentiële pathogenese die leidt tot een ulcus duodeni of een ulcus ventriculi. De prevalenties van precancereuze laesies zoals atrofische gastritis en intestinale metaplasie, waren hoger in ulcus ventriculi patiënten dan in ulcus duodeni patiënten. Dit is mogelijk gerelateerd aan het hogere risico bij ulcus ventriculi patiënten om maagkanker te ontwikkelen.

Bij patiënten met primaire maaglymfomen en patiënten met maagkanker werden gelijke frequenties van *H.pylori* (respectievelijk 61.5% en 53.8%) en non-*H.pylori* flora (respectievelijk 65.4% en 69.2%) gevonden. De prevalenties van *H.pylori*, gevonden met een immunohistochemische kleuring waren lager in vergelijking met de gegevens uit de literatuur en verschilden nauwelijks van de prevalentie in gezonde vrijwilligers van dezelfde leeftijd.

Bij patiënten met primaire maaglymfomen waren de prevalenties van *H.pylori* hoger, maar niet significant verschillend in de mucosa van het corpus (62.8%) ten opzichte van het antrum (45.5%). De dichtheid van *H.pylori* in het corpus van patiënten met een grootcellig maaglymfoom was hoger ten opzichte van patiënten met een kleincellig maaglymfoom evenals de prevalentie van non-*H.pylori* flora in tumorweefsel. Beide waarnemingen kunnen een gevolg zijn van verschillen in tumormassa en/of verschillen in de zuurgraad van de maag.

Bij de maagkanker patiënten werden geen significante verschillen gevonden

betreffende de aanwezigheid van *H.pylori* of non-*H.pylori* flora in relatie tot het type tumor of de histologische locatie hiervan. Twintig procent van de patiënten was positief voor *H.pylori* op basis van serologie maar negatief op basis van histologie. Deze bevinding ondersteunt de hypothese dat *H.pylori* een initiële rol zou kunnen spelen bij de ontwikkeling van maagkanker. Bij een kwart van de patiënten met maagkanker kon *H.pylori* niet worden aangetoond met behulp van immunohistochemie of serologie. In deze groep patiënten spelen andere factoren dan de aanwezigheid van *H.pylori* waarschijnlijk een rol in de pathogenese van maagkanker zoals de aanwezigheid van type A atrofische gastritis: dit type gastritis werd in 50% van de *H.pylori* negatieve maagkanker patiënten gevonden. Ook de aanwezigheid van relatief grote hoeveelheden non-*H.pylori* bacteriën, zoals aangetroffen bij mensen met maagkanker en bij mensen met een primair maaglymfoom, kunnen een rol spelen. Deze non-*H.pylori* flora zou kunnen bijdragen aan de ontwikkeling of progressie van maligniteiten in de maag door als antigene trigger te fungeren of door de productie van carcinogene of toxische metabolieten.

De rol van de totale bacteriële flora, *H.pylori* en non-*H.pylori*, in de pathogenese van gastrische maligniteiten vereist nader onderzoek. Hierbij is het gebruik van specifieke immunohistochemische kleuringen voor de histologische detectie van *H.pylori* in combinatie met een sensitieve serologische test noodzakelijk.

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Dankwoord

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Curriculum Vitae

Curriculum Vitae

Daisy Jonkers werd geboren op 2 december 1968 te Nederweert. In 1987 behaalde zij het VWO-diploma aan de Philips van Horne Scholengemeenschap te Weert. Aansluitend begon zij met de studie Gezondheidswetenschappen, richting Biologische Gezondheidskunde, aan de Universiteit van Maastricht. Zij haalde op 18 november 1992 haar doctoraal diploma en trad per 15 januari 1993 in dienst als assistente in opleiding bij de vakgroep Gastroenterologie van de Universiteit van Maastricht. In nauwe samenwerking met de vakgroepen Medische Microbiologie en Pathologie werkte ze aan het onderzoek dat beschreven is in dit proefschrift.

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