Genetic variation in Helicobacter pylori
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Citation for published version (APA):

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Chapter 1

General Introduction
Introduction

The discovery of *Helicobacter pylori* in 1983 by Warren and Marshall (1,2) has opened the new era for the peptic ulcer disease. The dogma “no acid, no ulcer” had to be revised and became “no *H. pylori*, no ulcer”. This chapter will review the literature with emphasis on the virulence factors of *H. pylori*.

Microbiology

*H. pylori* is a spiral-shaped, Gram negative microorganism with 4-6 unipolar, sheathed flagella. The bacterium measures 0.5-1.0 μm in width and 2.5-4.0 μm in length (3, 4). *H. pylori* is routinely cultured from antral and less frequently from corpus biopsies obtained from patients undergoing gastroscopy. The bacterium grows on freshly prepared blood agar plates (5) as well as in liquid medium (e.g. brucella broth, brain heart infusion medium) supplemented with serum or β-cyclodextrin (6). On solid media, small translucent colonies appear after two to four days of incubation under microaerophilic conditions (10% CO₂, 5% O₂, 85% N₂) at 37°C. When environmental conditions become unfavorable, e.g. by prolonged culture or exposure to oxygen, the spiral form slowly disappears and coccoid forms emerge. For a long time it was assumed that these forms might play a role in transmission of the bacterium. However, recent work by Küsters and co-workers (7) has shown that these coccoid forms are non-viable and this makes their role in transmission less likely. An important property of this bacterium is that it produces an abounding amount urease which can catalyze the break down of urea into carbon dioxide and ammoniumhydroxide, thereby neutralizing free protons. The presence of urea is obligatory for the survival of *H. pylori* in an acidic environment, because the organism is then able to survive a pH level of 1.5.

*H. pylori* can be identified by Gram’s stain and by positive oxidase, catalase and urease reaction (8). In histological sections the bacterium can be detected by modified Giemsa stain, Warthin-Starry silver stain or by incubation with specific antiserum. Since *H. pylori* can produce local and systemic antibodies after infected in human, the diagnosis of *H. pylori* infection can also be established through the detection of anti-*H. pylori* antibody in the serum (9-17). There are some commercially available diagnostic kits for non-invasive diagnosis of *H. pylori* infection. But whether they are suitable for different geographic regions (since the genotype of *H. pylori* is different in different geographic regions, see this thesis) are still need to be investigated.

Epidemiology

Infection with *H. pylori* occurs worldwide, but the prevalence varies per geographical region. It has been shown, that about 40% of children in China have acquired infection with *H. pylori* by the age of 10 years, and the prevalence rises with age to about 70% reached by the age of over 30 years (12, 18). In other developing countries, it was reported that about 50% of children are infected with *H. pylori* and infection rate increases with age up to 80-90% in adulthood (19-21). In developed countries less than 10% of the children are infected with *H. pylori* and the prevalence of infection increases to approximately 60% reached at the age of 60 years (19-21). The differences in prevalence are likely due to the socio-economic status, genetic and ethnic differences (22,
The mode of transmission of *H. pylori* is unknown. The most widely held hypotheses are that the organism is transmitted directly from person to person by human feces (fecal-oral spread) and gastric contents (gastric-oral spread). Improperly cleaned endoscopic equipment has been reported as cause of iatrogenic *H. pylori* infections (24). The handful of documented cases of acute *H. pylori* infections have all suggested gastric-oral routes of transmission. *H. pylori* has been cultured from vomitus, saliva, and diarrheal stools (25). There is evidence that *H. pylori*, by decreasing the gastric acidity, permits acid-sensitive gastrointestinal pathogens to pass through the stomach into the intestines (26). By thus increasing the risk for gastroenteritis, *H. pylori* may be favoring its own excretion and perpetuation.

**Links between *H. pylori* and Disease**

**Gastritis**

The first evidence that *H. pylori* causes gastritis came from two studies in healthy volunteers who ingested a culture of bacteria (27, 28). They developed epigastric pain with colonization of the antrum by *H. pylori* and acute inflammation. Eradication, either spontaneous or after treatment with antibiotics resulted in an improvement of gastritis. Nearly all persons who are infected with *H. pylori* develop gastritis, but in the majority of infected humans this gastritis is asymptomatic. Once the chronic phase has been reached, the infection probably persists for life.

**Peptic ulcer disease**

Among patients with duodenal ulcers the prevalence of antral *H. pylori* infection has consistently been found to be between 95 and 100% (29, 30). Although there is wide variation in the reported prevalences of *H. pylori* infection among patients with gastric ulcers, it has been argued that much of the variation is explained by selection criteria. If care is taken to eliminate drug-induced ulcers, then the prevalence approaches 100%. The gastritis associated with duodenal ulcer predominantly affects the antrum, and the chronic gastritis which accompanies gastric ulceration is characteristically a diffuse or pan-gastritis and frequently exhibits both multifocal glandular atrophy and intestinal metaplasia (31, 32). The relapse for both ulcers is significantly reduced after successful *H. pylori* eradication (33-36). The exact mechanisms of development of peptic ulcer disease are not completely understood and differences in pathogenesis of gastric and duodenal ulcer formation are under investigation.

**Gastric Cancer**

It is now well recognized that *H. pylori* is a major inducer of the multistep process that ultimately leads to gastric cancer (37, 38). Early acquisition in life appears to increase the risk of developing gastric cancer (39). Approximately 30% of the gastric cancers in the developed world and 50% in the developing world have conservatively been estimated to be attributable to *H. pylori* infection, although often higher percentages are quoted (40). Development of atrophic gastritis together with concomitant intestinal metaplasia appears to be the dominant step in the ultimate carcinogenic process leading to the intestinal type of adenocarcinoma (41).
Gastric MALT-lymphoma

The first evidence for an association between \textit{H. pylori} and primary gastric mucosa associated lymphoid tissue (MALT) lymphoma was shown in a retrospective study by Wotherspoon \textit{et al} who found that \textit{H. pylori} infection is present in 92\% of the patients with gastric MALT lymphoma (42). Further epidemiological evidence for an association between \textit{H. pylori} infection and gastric MALT lymphoma comes from a nested case-control study showing that individuals with \textit{H. pylori} infection had a six-fold higher risk for the subsequent development of gastric lymphoma. In contrast, there was no significant difference between patients with extra-gastric non-Hodgkin’s lymphoma prior \textit{H. pylori} infection and controls (43). The strongest evidence for the significance of \textit{H. pylori} in the pathogenesis of low-grade gastric MALT lymphoma is provided by clinical studies showing that cure of \textit{H. pylori} infection is followed by a complete regression of these tumors in most patients (44-48).

Virulence factors

Many factors contribute to the virulence of \textit{H. pylori} (49-59). Expressed by all isolates are factors required for colonization and survival in the human stomach. Most notable among these factors are the urease and flagella. Urease metabolizes urea to carbon dioxide and ammonia to buffer the gastric acid. Flagella allow the bacterium to move across the viscous gastric mucus and reach the more neutral pH in the deeper mucus layer. Knockout mutants of the urease or flagella genes are defective in colonization in a gnotobiotic piglet model of infection (60).

Once below the mucus, \textit{H. pylori} may adhere tightly to the underlying gastric epithelial cells. Several epithelial structures have been implicated in adhesion, including lipids, gangliosides, and sulfated carbohydrates, but to date, the adhesins on the bacterial surface that bind to the gastric epithelium are poorly understood (61, 62). The best-characterized \textit{H. pylori} adhesin is a protein (BabA) that binds the Lewis b blood group antigens on the gastric epithelium (63). Lewis blood group antigens have also been implicated in another variable aspect of \textit{H. pylori} pathogenesis; the lipopolysaccharide of some strains contains structures identical to the fucosylated Lewis X and Lewis Y blood group antigens expressed on the gastric mucosa. This antigenic mimicry may result in immune tolerance against antigens of the pathogen or in induction of autoimmune antibodies that recognize gastric epithelial cells, frequently observed in patients with chronic active gastritis (53).

One of the great challenges in \textit{H. pylori} research is to identify the bacterial virulence factors that account for the remarkable range of clinical outcomes. The concept that the \textit{H. pylori} genotype could be an important determinant of the severity of infection emerges from studies demonstrating considerable nucleotide-level diversity among independent \textit{H. pylori} isolates (64) as well as from studies showing the diversity of several bacterial phenotypes. Of particular interest in this context are two traits that are polymorphic in the \textit{H. pylori} population: production of a protein (VacA) that causes vacuoles in cultured mammalian cells (65) and the presence or absence of a gene called \textit{cagA} (58, 66). Since this thesis mostly deal with these two traits, this chapter will focus on the vacuolating
cytotoxin and the cagA gene and on their possible role in the virulence of *H. pylori* infections.

In 1988, Leunk and colleagues found that 50% of the *H. pylori* strains isolates produce a proteinaceous *H. pylori* factor that induces the formation of large cytoplasmic vacuoles in cultured mammalian cells and suggested that this effect was caused by a secreted *H. pylori* cytotoxin (67). Later on, Figura and others reported that 60% of *H. pylori* isolates from patients with peptic ulcer disease seem to be cytotoxin positive (Tox+), whereas only 30% of isolates from patients without ulcers are Tox+ (68-70). These data suggest that VacA is a determinant or marker of virulence. Vacuolation of epithelial cells has been detected in gastric biopsy specimens from *H. pylori*-infected persons (71, 72), but remarkably, prominent vacuolation similar to that observed in cell culture systems is not usually seen.

VacA was purified based on its capacity to induce cell vacuolation and was found to migrate on a SDS polyacrylamide gel under denaturing conditions as a 90-kD protein (73). It causes vacuolar degeneration of target cells by interfering with intracellular membrane fusion. The vacuoles appear to be a hybrid between lysosomal and late endosome compartments, and their generation requires the vacuolar adenosine triphosphate-dependent proton pump and the small guanosine triphosphate-binding protein Rab7. More recently, the toxin has been shown to reduce transepithelial resistance by loosening tight junctions (74). VacA is a potent antigen, and its vacuolating activity can be neutralized by serum antibodies from immunized rabbits (73) or by serum antibodies from *H. pylori*-infected humans (75, 76).

The vacA gene, which encodes the vacuolating cytotoxin, was cloned by several groups using degenerate oligonucleotides that were designed based on partial amino acid sequence data (55, 57, 77, 78). DNA sequence analyses identified a single open reading frame whose inferred product corresponds to a protein ~140 kD in size. Comparison of experimentally determined VacA protein sequences with the deduced VacA sequence indicate that a 140-kD VacA protein undergoes proteolytic cleavage at both its N- and C-termini, to yield the mature secreted ~90-kD VacA protein, a 33-amino-acid signal sequence, and a C-terminal fragment. DNA sequence analysis of vacA genes from several representative Tox+ and Tox− *H. pylori* strains has identified several families of vacA alleles (57, 79). Two families of alleles (m1 and m2) are distinguished by striking sequence differences in the mid-portion of vacA, and three families (s1a, s1b, and s2) are distinguished by differences in vacA signal sequences (79). *H. pylori* isolates with type s2 vacA signal sequences do not produce detectable vacuolating activity in *vitro*, whereas ~60% of the isolates with vacA s1 alleles produced detectable vacuolating activity (79). Analysis of 59 different *H. pylori* isolates has established five of the six possible combinations of vacA signal sequence and middle-region types (s1a/m1, s1b/m1, s1a/m2, s1b/m2, and s2/m2, but not s2/m1) (79). Vacuolation and more severe clinical manifestations seem to be associated with particular vacA alleles. *H. pylori* strains with the signal sequence allele s1a are associated with higher levels of vacuolating cytotoxin activity, enhanced gastric inflammation, and duodenal ulceration than are the s1b or s2
alleles. The m1 middle region allele is associated with higher levels of toxin activity and more severe gastric epithelial damage than is the m2 allele (79, 80).

Early studies showed that ~60% of H. pylori isolates from patients in the United States and Western Europe produce an immunodominant 120-kD to 140-kD protein that later came to be called CagA, whereas the other 40% of strains do not (81, 82). Expression of CagA was significantly more common among Tox+ strains than Tox- strains (82). Subsequently, the gene for CagA protein was cloned from expression libraries, sequenced, and shown to be present in strains that produced the ~120-kD to 140-kD protein and absent from strains that did not produce CagA protein (58, 66). The cagA gene contains several different sets of repeating units that are 15 to 48 nucleotides in length and vary in copy number among isolates. Differences in the copy number of these repeating units account for the size variation (120 kD to 140 kD) in CagA proteins of different isolates (58, 66).

Histological studies have shown that mucosal neutrophil inflammation is typically more severe in persons infected with cagA+ H. pylori strains than in persons infected with cagA- strains (83). Wild-type cagA+ H. pylori strains also induce higher levels of gastric inflammation in the mouse stomach than do wild-type cagA- strains (84, 85). cagA+ H. pylori infections of humans are characterized by higher mucosal levels of proinflammatory cytokines, such as interleukin-8 (IL-8; a neutrophil chemotactic factor) than are cagA+ H. pylori infections (86). Similarly, cagA+ strains induce higher levels of IL-8 synthesis by epithelial cells in vitro than do wild-type cagA- strains (87-89). Further studies of epithelial cell IL-8 synthesis in vitro have shown that products of genes linked to cagA, rather than CagA itself, help trigger this response (90).

The capacity of cagA+ strains to induce a severe inflammatory response probably contributes to an increased risk of clinically evident disease in patients infected with these strains. Studies from U.S. and Europe have shown that serum and gastric mucosal antibody responses to CagA are detected more often in H. pylori-infected patients with ulcer disease than in comparable patients without ulcers (14, 58, 82, 83). The presence of serum anti-CagA antibodies is also associated with an increased risk of atrophic gastritis, intestinal metaplasia (91), and gastric adenocarcinoma (92, 93).

Experiments in several laboratories (90, 94, 95) have shown that cagA is at one end of a large (20-kb to 40-kb) DNA segment that is present in all cagA+ strains tested and absent from cagA- strains. Multiple genes in this region have been identified that are each required for cagA+ H. pylori strains to induce IL-8 synthesis by epithelial cells (90, 95). In contrast to the need for these genes in cytokine induction, mutational tests have shown that cagA is not required, and its function remains unknown.

Vaccines may end the Coexistence of Man and H. pylori

The discovery of the infectious nature of peptic ulcer has dramatically changed the medical approach to this disease. Although combinations of antibiotics are effective in eradicating H. pylori, strains resistant to antibiotics are already emerging (96), decreasing the efficacy of the currently used triple therapy. Furthermore, antibiotics cannot be used
to eradicate the infection from the whole population, especially in developing countries. It is therefore predictable that although antibiotics are a good solution for individual treatment of disease, they will not represent a definitive solution for society. Hence, vaccination—the most effective medical practice in controlling infectious diseases on a global scale—may represent the ultimate solution.

The challenge to develop a vaccine has been particularly successful in mouse models with either the *H. pylori*-related species-*Heliocobacter felis* (97)—or the mouse-adapted *H. pylori* that mimics human infection (98, 99). Vaccine-induced protection from infectious challenge and eradication of established infection have been proved with many antigens, including whole inactivated cells, bacterial lysates, and several purified antigens such as urease, heat shock protein, VacA, CagA and catalase (97, 100). The most successful approach has been mucosal immunization with adjuvants such as cholera or *Escherichia coli* enterotoxins or the genetically detoxified derivative, LTK6, mixed with one or more of the antigens (101). Since we have found that, based on the sequence analyses of *cagA* and *vacA* genes, *H. pylori* vary with geographic region (this thesis), antigenic epitopes of immunogenic proteins of *H. pylori* from different geographic regions may also vary. While many of these animal models are using either CagA or/and VacA of *H. pylori* from Western countries as antigens, the effectiveness of administered CagA and/or VacA of *H. pylori* from different human populations (e.g. Asia) need to be evaluated.

Animal models have their limits, however. Well-designed clinical trials are now required to answer whether the promising results obtained in animals apply to humans. If successful, vaccination may indeed be able to end the coexistence of humans and *H. pylori*.

**Scope of this thesis**
The two main virulence factors of *H. pylori*, the CagA protein encoded by cytotoxin associated gene A (*cagA*) and VacA encoded by vacuolating cytotoxin gene (*vacA*) are mainly studied in Western countries using *H. pylori* isolated from patients from these countries. The prevalence of these virulence factors in *H. pylori* from East Asia, their relationship with the disease outcome in this population and whether these factors can be used as markers for the diagnosis of gastroduodenal diseases or as predictor of risk factors in the development of different *H. pylori*-related diseases are unknown. Answers to these questions are very important, not only to increase our knowledge of the pathogenesis of *H. pylori*-related diseases, but it will also have a powerful practical use for clinicians and scientists involved in the development of new markers for the diagnosis of *H. pylori*-related diseases and in the identification of targets for new drugs and vaccines. In addition, it will also help us to understand the *H. pylori* population genetics.

The aim of this thesis is to get more insight into the genetic differences between *H. pylori* isolated from patients from different geographic regions in relation with *H. pylori*-related diseases. This research can be divided in three main parts: i) the prevalence of *cagA* positive *H. pylori* in China and its relationship with the disease; ii) the prevalence of vacuolating cytotoxin production and the distribution of the different *vacA* alleles in
Chinese *H. pylori* as well as their relationship with the disease; iii) Comparison of Chinese *H. pylori* with the *H. pylori* from Western and Asian countries with regard to both virulence factors and non-virulence factors at the DNA sequence level.

The strong association between infection with *cagA*-positive *H. pylori* and PUD are mainly reported from North American and Western European populations. Whether this association also applies to *H. pylori* isolated from Chinese patients was studied in chapter 2. Different techniques, including PCR, were used to detect *cagA* of *H. pylori*. Remarkably, we found that the primers which were used to detect the *cagA* gene in *H. pylori* from the Netherlands do not work well with *H. pylori* from China, indicating differences between the sequence of *cagA* in Chinese *H. pylori* and *cagA* in Dutch *H. pylori*. In chapter 3, this hypothesis was investigated by sequencing part of *cagA* and *glmM* of 24 randomly chosen *H. pylori* isolates from 12 Dutch (14 isolates) and 10 Chinese patients. The random amplified polymorphic DNA (RAPD)-PCR was also employed to compare the *H. pylori* from these two human populations. Whether the aforementioned correlation between vacuolating cytotoxin activity, specific *vacA* alleles and *H. pylori* related diseases also applies to *H. pylori* from other countries is unknown. In chapter 4, the phenotype and the genotype of the *vacA* of Chinese *H. pylori* and their relation with clinical manifestations were studied. The association of specific *vacA* alleles with disease are mainly from U.S., and the results from the aforementioned chapters indicate that the distribution of *H. pylori* genotypes and the association with disease may be different in other geographic areas. Therefore, the aim of chapter 5 was to establish whether *vacA* types among *H. pylori* isolates from Dutch patients were associated with disease.

Multilocus enzyme electrophoresis of six loci of *H. pylori* indicated no linkage disequilibrium between the different loci (102). Similarly, analyses of sequences from fragments of the *vacA*, *flaA* and *flaB* genes indicated "free recombination" among isolates from Canada and Germany (103), i.e. no clonal associations were detected, even among pairs of polymorphic sites within single genes. These seemingly contradictory interpretations are from limited data available on the population genetics of this organism. Analysis of a more global collection of *H. pylori* might reveal geographic associations that were lacking in a limited sample. Furthermore, alleles of cytoplasmic housekeeping genes are more likely to be selectively neutral and therefore uniform than is the case of genes that might be under selective pressure due to their association with virulence (*cagA*, *vacA*, *flaA*, *flaB*). In chapter 6, we analyze the sequence variability of both housekeeping and virulence-associated genes from *H. pylori* strains isolated in several continents and show that weakly clonal groupings can be identified in *H. pylori* despite a rich history of interstrain recombination.
References


