Studies on bacterial transmission pathways in a high endemic area, with a focus on *Helicobacter pylori*

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“Time makes more converts than reason.”

- Thomas Paine

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Abstract

Even though half of the world's population is infected with *Helicobacter pylori*, which causes gastritis, peptic ulcer and gastric cancer, the transmission routes of these bacteria remain unknown despite extensive epidemiological studies. Enterotoxigenic *Escherichia coli* (ETEC) and *Vibrio cholerae* are two of the most common causes of acute watery diarrhea in developing countries. The main aim of this thesis was to study transmission pathways of these bacteria, with a focus on *H. pylori,* through analyses of clinical and water samples from Dhaka, Bangladesh, an area with high prevalence of gastrointestinal diseases.

To determine the bacterial numbers in clinical and water samples we developed highly sensitive quantitative real-time PCR assays targeting specific and conserved virulence genes of *H. pylori* (*cagA*, *flaA*, *glmM*, *hpaA*, *ureA* and *vacA*), ETEC (*eltA* and *estB*) and *V. cholerae* (*ctxB* and *tcpA*). The assays were used for quantification of bacterial DNA and reverse-transcribed gene transcripts.

Twenty-six of 39 (67 %) drinking and environmental water samples from a poor area in Dhaka were positive by real-time PCR for ETEC, whereas all 75 drinking and environmental water and 21 drinking water biofilms from the same location were negative for *H. pylori*, suggesting that ETEC may be waterborne while *H. pylori* is not.

H. pylori transmission during epidemics of gastroenteritis was then explored by analyzing vomitus and stool samples collected from diarrhea patients admitted to the ICDDR, B hospital in Dhaka. All samples were positive for *V. cholerae*, with higher numbers in stool (median 2.5 x 10^6 genomes) than vomitus (median 2.7 x 10^4 genomes) and a strong correlation between DNA real-time PCR and quantitative culture. Analyses for *H. pylori* showed that 23 of 26 (88 %, median genome number = 4.35 x 10^5 ml^{-1}) vomitus and 17 of 23 (74 %, median genome number = 7.33 x 10^2 ml^{-1}) stool samples were positive in real-time PCR, but *H. pylori* could not be isolated by culture. The results indicate that high numbers of *H. pylori* are shed in vomitus during acute gastroenteric disease and indicate that *H. pylori* may be transmitted by this route. To establish possible infectivity of these bacteria, the gene expression of *H. pylori* in vomitus, stool and biopsies from infected individuals and in in vitro cultures was analyzed. Vomitus, biopsies and in vitro cultures showed high expression of *cagA*, *flaA* and *ureA* and lower expression of *hpaA* and *vacA*, whereas no expression was detected in diarrheal stool. Expression analyses of the same genes in a C57Bl/6 *H. pylori* strain SS1 infection mouse model showed a similar relative transcription pattern as in biopsies, in vitro cultures and vomitus and that expression is up-regulated during exponential growth.

In conclusion, our results suggest that *H. pylori* may be disseminated through vomitus during outbreaks of gastrointestinal infections in Bangladesh and that waterborne transmission is less likely whereas waterborne transmission of ETEC may occur. Furthermore, the studies indicate that experimental murine infection and vomitus from *H. pylori* infected subjects may be suitable models of *H. pylori* virulence gene expression in vivo.

Keywords: *H. pylori*, enterotoxigenic *E. coli*, *V. cholerae*, real-time PCR, transmission pathways, gastroenteritis, mouse infection models, bacterial gene expression.
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Original papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals (I-IV):

I. Lothigius Å., **Janzon A.**, Begum Y., Sjöling Å., Qadri F., Svennerholm A.-M. and Bölin I. Enterotoxigenic *Escherichia coli* is detectable in water samples from an endemic area by real-time PCR.
Journal of Applied Microbiology 2008 104 (4):1128-1136.

II. **Janzon A.**, Sjöling Å., Lothigius Å., Ahmed D., Qadri F. and Svennerholm A.-M. Failure To Detect *Helicobacter pylori* DNA in Drinking and Environmental Water in Dhaka, Bangladesh, Using Highly Sensitive Real-Time PCR Assays.
Applied and Environmental Microbiology May 2009 75: 3039-3044

III. **Janzon A.**, Bhuiyan T., Lundgren A., Qadri F, Svennerholm A.-M. and Sjöling Å.. Presence of high numbers of transcriptionally active *Helicobacter pylori* in vomitus from Bangladeshi patients suffering from acute gastroenteritis.
Submitted

IV. **Janzon A.**, Svennerholm AM and Sjöling Å.
Helicobacter pylori virulence gene expression in a mouse model.
Manuscript

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Abbreviations

AMP	Adenosine monophosphate
BabA	Blood group binding protein A
BHI	Brain heart infusion
cag PAI	Cytotoxin associated gene pathogenicity island
CagA	Cytotoxin associated gene A
cDNA	Complementary DNA
C_P	Crossing point
CT	Cholera toxin
C_T	Threshold cycle
DNA	Deoxyribonucleotide acid
E. coli	*Escherichia coli*
ELISA	Enzyme-linked immunosorbent assay
ETEC	Enterotoxigenic *Escherichia coli*
Fla	Flagellin
GlmM	Phosphoglucosamine mutase
GMP	Guanosine monophosphate
H. pylori	*Helicobacter pylori*
HpaA	*Helicobacter pylori* adhesin A
HP-Nap	*Helicobacter pylori* neutrophil-activating protein
ICDDR, B	International Centre for Diarrhoeal Disease Research, Bangladesh
Ig	Immunoglobulin
LB	Luria-Bertani
LPS	Lipopolysaccharide
LT	Heat-labile enterotoxin
mRNA	Message RNA
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RNA	Ribonucleotide acid
rRNA	Ribosomal RNA
RT	Reverse transcriptase
SabA	Sialic acid binding protein A
spp.	Species (plural)
SS	*Salmonella Shigella*
ST	Heat-stable enterotoxin
T4SS	Type 4 secretion system
TCBS	Thiosulfate citrate bile sucrose
TCP	Toxin co-regulated pilus
TTGA	Taurocholate-tellurite-gelatin agar
UBT	Urea breath test
UreA	Urease subunit A
V. cholerae	*Vibrio cholerae*
VacA	Vacuolating cytotoxin A
VBNC	Viable but not culturable

Introduction

Helicobacter pylori

Helicobacter pylori, one of the most common human bacterial pathogens, causes a chronic infection of the human gastric mucosa (Figure 1). The species was not isolated until the early 1980s (102), despite some earlier observations of human gastric bacteria. Since its discovery, there has been a remarkable amount of research on this bacterium, driven to a large extent by the fact that it is the primary etiologic cause of both peptic ulcers (84) and gastric tumor disease (1). The fact that it is the only known bacterium naturally and persistently residing in the human stomach has no doubt increased the interest in this pathogen even further. However, despite more than two decades of clinical and basic investigations, its primary transmission pathway is still not known and our knowledge of the initial stages in its colonization of the human gastric mucosa is also scarce. The main subject of this thesis is studies on *H. pylori* transmission (Figure 1) in Bangladesh, a high endemic country. The thesis will also describe limited studies on the enteric diarrheagenic pathogens enterotoxigenic *Escherichia coli* (ETEC) and *Vibrio cholerae* with relevance for *H. pylori* transmission. Finally, comparisons of the expression of selected *H. pylori* virulence genes between human biopsies, a murine *H. pylori* infection model and suggested sources of new *H. pylori* infections are also described.

Epidemiology and transmission

Although the transmission pathway of *H. pylori* is not conclusively determined, other aspects of its epidemiology are well characterized. Today, the prevalence of *H. pylori* is very high in most low and middle income countries (86), whereas there is increasing evidence of its slow eradication in high income countries (53), although the mechanisms behind this are unknown. The prevalence ranges from 85-90 % in for instance Bangladesh (3, 98) to less than 20 % in Australia and Sweden. In high endemic areas, *H. pylori* is acquired in early childhood (17, 49, 110, 111), and its prevalence is high in all age-groups (125). In high income countries, *H. pylori* is probably also acquired in early childhood in most cases (86), although there is some evidence to the contrary (85, 134, 169). Prevalence then increases with age in most developed countries (79, 82, 135, 139). There is some geographic variation in the frequency of *H. pylori* related diseases (142), but in general the bacterium causes gastric or duodenal ulcers in approximately 10-15 % of those infected (151) and gastric cancer in another 1-2 % (81).

Several different transmission pathways have been proposed for *H. pylori*. Studies aiming to determine the transmission routes have used very different methodologies, including epidemiological surveys investigating risk factors (71, 76, 106, 120, 166), molecular detection methodology in a vast range of suspected sources and bioinformatic and statistical evaluations of clonal relatedness of strains isolated from within families and from small communities (39, 75, 144). Not surprisingly, the studies have reached rather different conclusions, but in general two main hypotheses can be distinguished. The first of these hypotheses highlight the putative importance of external reservoirs, usually either drinking or environmental water sources or foodstuffs. Many of these studies report detection of *H. pylori* using molecular methods or in some cases successful isolation in samples of water (15, 24, 28, 29, 50, 59, 64, 66, 67, 104, 105, 107, 119, 130, 168), food (41, 51, 129), or domestic and other animals living in close proximity to humans (40, 41, 51, 56, 129). There are also many epidemiological studies that have identified especially drinking water sources as a major risk factor for *H. pylori* infection (71, 76, 94, 95, 120). The second of the two hypotheses instead propose that *H. pylori* is spread directly from person to person, usually within the family or close communities (74, 106, 126, 150, 166). This hypothesis is supported by studies using epidemiological surveys or bioinformatic analyses of strain relatedness. There are also many studies using molecular methods to detect traces of *H. pylori* in fecal matter, saliva and dental plaque (42, 70, 130), although these data may be used to support either hypothesis. To reconcile the two hypotheses it has been proposed that transmission may occur via several distinct routes, with different predominance in different geographic areas (144), i.e. that water and other external sources of *H. pylori* are common in developing countries with low standards of drinking water and hygienic practices, whereas person-to-person transmission is predominant in developed countries. As of today, the relative importance of the different proposed pathways remains to be conclusively shown. Unfortunately there are no reports of mathematical models of *H. pylori* transmission routes. Such models have helped researchers to elucidate transmission patterns and risk factors of other important pathogens such as *V. cholerae* (72, 73), *Trypanosoma cruzi* (35) and HIV (22), and might provide novel ideas for *H. pylori* as well.

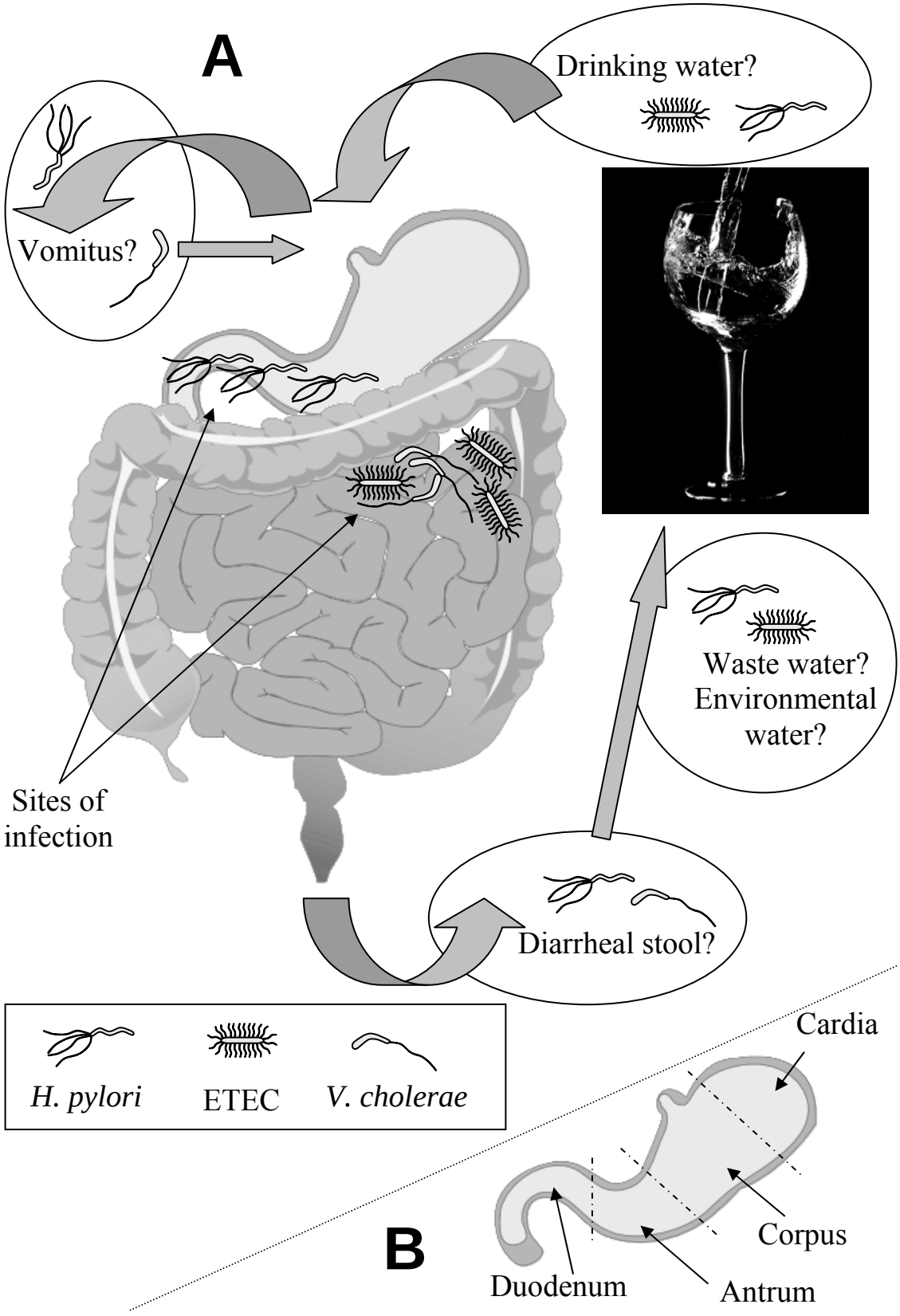


Figure 1: A. Schematic picture of the gastrointestinal system with the infection sites of *H. pylori*, ETEC and *V. cholerae* and suggested sources of infection investigated in this thesis. B. The different compartments of the human stomach and duodenum.

In addition to the above controversies, it is unknown how *H. pylori* is shed from an infected individual. Most reports favor fecal shedding (86), but gastric (vomitus) (14, 87, 91, 124, 155) and oral (saliva, dental plaque) (42, 70, 101, 163) shedding have also been suggested. Existing data are mostly from molecular studies but in some cases from epidemiological surveys or from successful isolation of *H. pylori* (86, 91, 92, 97, 108, 124, 126). However, the evidence for any of these routes is scarce, and the fecal route often seems to be favored by analogy with enteric pathogens.

Microbiology

Since *H. pylori* was isolated in 1983, several other *Helicobacter* spp. have been described, as of today there are at least 44 species. The genus is generally divided into gastric and enteric *Helicobacter* spp., and most of the different species are associated with disease in humans or other hosts. In humans, the only known gastric helicobacters are *H. pylori* and *H. heilmannii*, but there are several enteric species, such as *H. cinaedi*, *H. fennelliae* and *H. pullorum*. Some helicobacters are also known to infect the liver, such as *H. hepaticus* and *H. pullorum*. The *Helicobacter* spp. and the *Wolinella* genus (only known member is *Wolinella succinogenes*, which colonizes the bovine rumen) form the *Helicobacteraceae* family. *Helicobacteraceae* together with the *Campylobacteraceae* family, to which *Campylobacter* spp. and *Arcobacter* spp. belong, are the two most well characterized families of the epsilonproteobacteria, a class of Gram-negative spiral- or rod-shaped bacteria. Several of the members of this class are known to cause gastroenteric diseases in humans and animals, such as e.g. *Campylobacter jejuni*, *C. coli*, *C. fetus* and *Arcobacter butzleri*.

The physiology of *H. pylori* has been relatively well studied. *H. pylori* is a fastidious, microaerophilic and capnophilic, curved or spiral-shaped bacterium with a temperature optimum between 34 and 40 °C. For in vitro growth, it requires an atmosphere with 5-10 % CO_2 and 2-5 % O_2 and complex media. It cannot replicate in minimal media or under aerobic conditions (86). In response to suboptimal conditions, the normally spiral-shaped *H. pylori* rapidly transform into small coccoid cells (Figure 2). It is presently unknown whether the coccoids are viable, but they can not be cultured in vitro. However, some studies have shown evidence for reversion to the actively dividing spiral shape in animal models (27, 30, 148). *H. pylori* is propelled by 2-6 unipolar sheathed flagella (52) consisting of two copolymerized flagellin subunits (FlaA and FlaB) (78), and it is highly motile with a cork-screw motility which is thought to allow the bacteria to penetrate the thick mucus layer that is protecting the human gastric epithelial lining from the highly acidic lumen. *H. pylori* itself is not acidophilic,

but tolerates low pH with a urease enzyme which allows it to maintain a neutral intracellular pH in acidic environments (13, 112, 157, 158) through production of ammonia and gaseous carbon dioxide from urea (25) that is released from the epithelial cells. The ammonia production may also serve as a nitrogen scavenging process (112, 167).

Pathogenicity and virulence factors

H. pylori causes gastritis, gastric and duodenal ulcers, gastric adenocarcinoma and gastric mucosa associated lymphoid tissue (MALT) lymphoma (86). Several *H. pylori* virulence factors contribute to *H. pylori* colonization and thus indirectly or directly also to the above pathologies, although host and environmental factors undoubtedly have a large role in disease development as well. In general, the most important virulence factors of *H. pylori* are the ones that confer its key pathogenic properties; acid tolerance, ability to penetrate mucus, adhesion to epithelial and other cells and manipulation of host immune responses and cell physiology. These properties are mediated mainly by urease, flagella, adhesins and exported and cell surface expressed antigens, respectively. Urease and flagella are discussed briefly above, and the main examples of the other categories follow below.

Many *H. pylori* strains carry a type IV secretion system (T4SS) , which is encoded in the *cag* pathogenicity island (*cag* PAI) (5, 31, 33) together with other virulence associated genes such as the cytotoxin associated gene A (*cagA*) (36). The presence of *cagA*, which is found in approximately 50 to 70 % of *H. pylori* strains (32, 38), is often used as a marker for presence of the *cag* PAI. *cag* PAI positive strains are associated with severe gastritis (18, 83), although *cag* PAI negative strains have been found also in ulcer and cancer patients. CagA, the product of *cagA*, is translocated into epithelial cells together with peptidoglycan from the bacterial cell wall and possibly other molecules by the T4SS (8, 34, 123), where it interferes with host kinases (8, 123, 145). Inside the host cells CagA then induces morphological changes (113), whereas the peptidoglycan triggers a proinflammatory response via the NOD1/NFκB pathway.

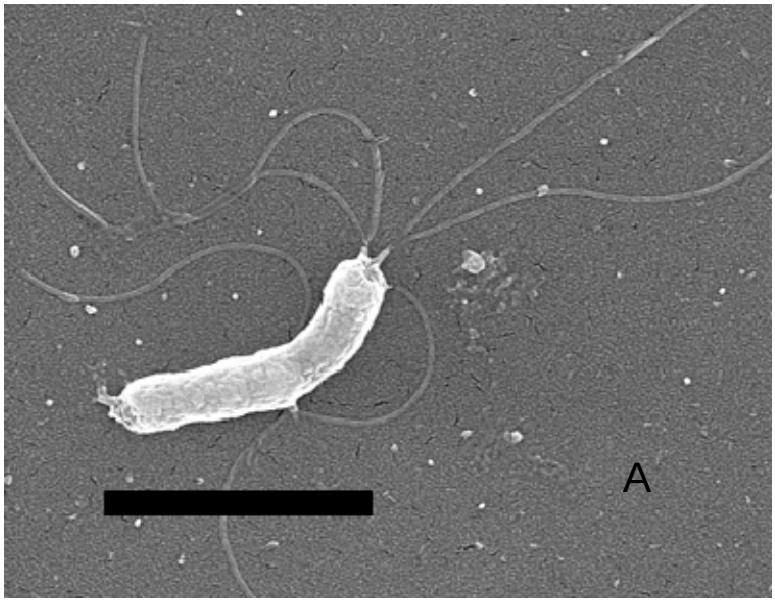
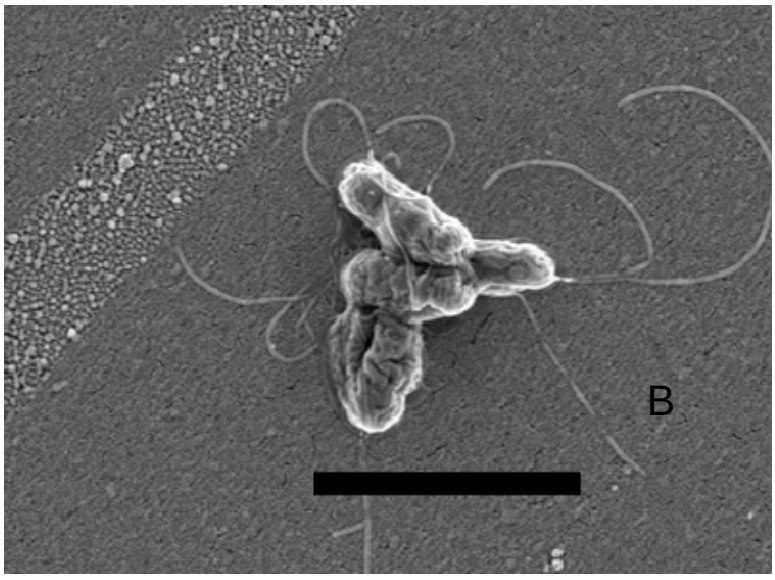
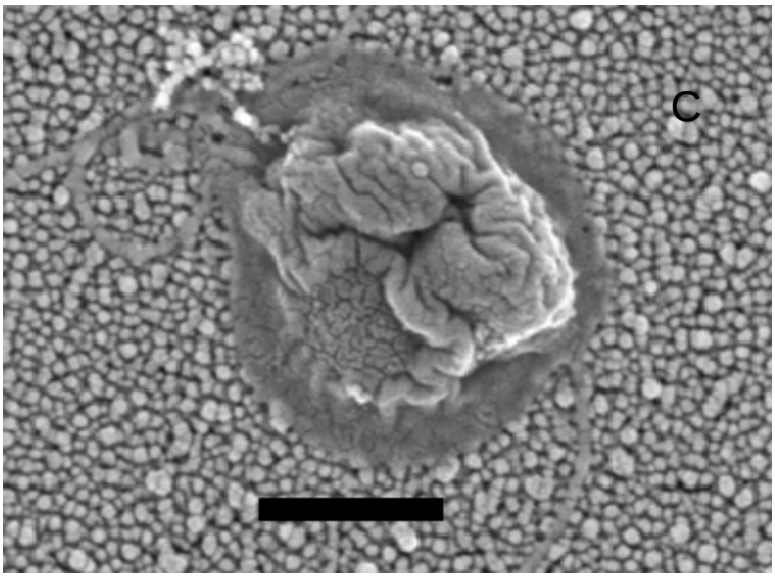


Figure 2:
Electron micrographs of *H. pylori* spiral-shaped and coccoid morphologies:

A. A single *H. pylori* cell with curved morphology from a 24 h microaerophilic culture in Brucella broth with 5 % calf serum at 37 °C. Magnification= 20000 times, bar = 2 µm.



B. Group of temperature-, nutrient-, and oxygen-stressed *H. pylori* cells with coccoid or nearly coccoid morphologies after suspension in PBS for 48 h at 4 °C. Magnification= 20000 times, bar = 2 µm.



C. A single *H. pylori* cell with coccoid morphology treated as in B. Magnification= 55000 times, bar = 0.5 µm.

In contrast to the *cag* PAI, all *H. pylori* strains carry the vacuolating cytotoxin A (*vacA*) gene. However, only some 50 % of the strains secrete the protein and the vacuolating properties of the protein seem to vary greatly between different strains (12, 37). The differences are due to sequence variation in the signal (s) and middle (m) regions of the gene which occur in two variants each: s1, s2, m1 and m2. The s1/m1 gene variant seems to be more virulent than other variants (12). The protein derives its name from its ability to cause massive vacuolization in in vitro grown epithelial cell lines (37), but this has not been observed in vivo. Instead, VacA in vivo has been suggested to form membrane channels in the epithelial cell membranes (114), which leads to release of urea and anions from the epithelial cells.

The neutrophil activating protein of *H. pylori* (HP-Nap) was named after its reported capacity to induce host neutrophils to produce and secrete reactive oxygen species (48, 171). Based on sequence homologies of the HP-Nap gene (*napA*) to bacterial ferritins (48), iron scavenging proteins, it has also been proposed to be involved in iron uptake and/or storage. Since the majority of studies on HP-Nap have been performed in vitro, its precise role in vivo remains elusive. However, it is well established that neutrophils are recruited to and penetrates the gastric mucosa during *H. pylori* infection (102, 133), suggesting that HP-Nap may be involved in manipulating either the neutrophil recruitment or response.

The *Helicobacter pylori* adhesin A (HpaA) is another elusive antigen that is believed to contribute to *H. pylori* infection and possibly also virulence. This lipoprotein is unique to *H. pylori* and even lacks homologues in other helicobacters except for *H. acinonychis*. It has been shown to be essential for colonization of mice (26), but its precise function in vivo is still unknown. It was initially proposed to be an adhesin (hence the name) binding to sialic acid, although this has been challenged. Its cellular location is also in doubt, with different studies showing location in the cytoplasm, flagellar sheath and outer membrane (20, 69).

Blood group antigen binding (BabA) protein and Sialic acid binding (SabA) protein are the two most well characterized proteins in the *Helicobacter pylori* outer membrane protein (Hop) family and both are adhesins that allow the bacteria to attach to epithelial and other cells. BabA binds to fucosylated Lewis b (Le^b) blood group antigens on the host cells (23, 68) and SabA binds to various sialylated glycoconjugates, primarily sialyl-Lewis a (Le^a) and sialyl-Lewis x (Le^x) antigens (99, 138). Both of the molecules have been suggested to be important for colonization (68, 93, 99). These two and other adhesins help the bacteria to manipulate the host environment by triggering cell surface receptors or through cell contact which allows the T4SS to transfer molecules to the host cells.

Lipopolysaccharides (LPS) are glycolipids with very strong toxic and immunostimulating properties that are found in the outer membrane of Gram-negative bacteria. All LPS molecules share the same basic architecture with three different regions. The innermost region is a lipid moiety (lipid A) which anchors the LPS in the outer membrane and is usually highly toxic. The middle region is an oligosaccharide with 10-15 sugars. The outer region is called the O-specific chain, consists of multiple repeats of oligosaccharide units and is highly variable even within bacterial species. *H. pylori* LPS has much lower toxicity than for instance LPS from *Enterobacteraceae* (19, 118), which may contribute to *H. pylori* colonization. Furthermore, the fucosylated O-specific chain of *H. pylori* LPS mimics various human blood group Lewis antigens (9-11, 115). The advantage for *H. pylori* of this molecular mimicry is not determined, but it has been suggested to contribute to adhesion or immune evasion (116) and even to induce autoimmunity in the host (7).

Immunology and vaccine development

During the chronic *H. pylori* infection, the host immune system mounts a vigorous systemic and mucosal response with associated gastritis. The mucosal response is characterized by infiltration of neutrophils, activation of dendritic cells and recruitment of B and T cells. Neutrophils are recruited to the gastric mucosa and activated both by bacterial factors and by chemokines (159) while dendritic cells are activated by *H. pylori* antigens. The T cell response consists mainly of Th1 polarized CD4+ T helper cells even though the infection is non-invasive. Serum IgA, IgG and IgM antibodies are evidence of the B cell response. However, neither adaptive nor innate natural immune responses are able to clear the infection. The inflammation is even believed to be beneficial for *H. pylori* through release of nutrients, although inflammation is important for eradication in animal models. The lack of sterilizing immunity has instead led to the conclusion that *H. pylori*, through the help of its many virulence factors, is able to adjust the immune responses to its advantage. This manipulation may for instance involve recruitment of regulatory T cells (Treg), which are abundant in the infected mucosa.

However, several studies have suggested that it may be possible to induce protective immunity by for instance vaccination, most probably through a combination of cellular and innate responses (4, 46). Many therapeutic and prophylactic vaccine candidates have consequently been evaluated in experimental studies (4, 46, 54, 58, 100, 121, 122, 131, 154, 162). The main candidates so far have largely been the virulence factors discussed above, alone or in different combinations and together with various adjuvants. Whole cells and whole

cell lysates of *H. pylori* and related helicobacters have also been tested. Despite moderate successes in animal models, no successful human clinical vaccine trial has been carried out.

Diagnostics and identification techniques

Several rather different techniques are used to diagnose *H. pylori* infection or to detect its presence in various sample types. In clinical settings, the Urease Breath Test (UBT) is often used as a rapid method to detect active infection. In this test, the patient ingests urea labeled with either carbon-13 or carbon-14 isotopes. After 10-30 min the presence of the labeled isotope is measured in exhaled breath carbon dioxide and a positive test indicates that the urea was metabolized by *H. pylori* urease. Serological tests and Western blots are also common. Various stool antigen tests are often used clinically to measure the amount of *H. pylori* specific antigens in fecal matter by different immunosorbent assays, usually ELISA. These tests are commercial and the target antigens are not revealed by the manufacturers. Other established methods are histology and culture from gastric and duodenal biopsies; methods that are often used if *H. pylori* infection is suspected based on results from rapid and non-invasive tests such as UBT or stool antigen tests. Culture and histology are arguably considered "gold standards", but they are labor intensive and invasive. Histology has the additional advantage of providing information on inflammation and atrophy, although it can only be performed by a trained pathologist. Biopsy culture on the other hand isolates the bacterial strain, which may provide additional data on its characteristics, such as *cag* PAI positivity. Several different solid media are used for culture of *H. pylori* from biopsies, among the more common ones are Brain Heart Infusion (BHI) agar, Chocolate agar, Brucella agar and horse or sheep blood agar. The media are always supplemented with 2-4 antibiotics; trimethoprim, vancomycin, cephalosporins and polymyxin B are among the most common. Sometimes the media are also supplemented with serum or β-cyclodextrin, which is believed to inhibit the effect of toxic byproducts produced by *H. pylori* during growth. *H. pylori* colonies are then identified after 3-7 days culture in microaerophilic conditions by visual inspection for grey, translucent, pinpoint colonies. Subsequent biochemical tests for positive oxidase, catalase and urease reactions are routine. Finally, a plethora of different PCR methods have been used to detect *H. pylori* DNA both in clinical specimens, such as gastric or duodenal biopsies, stool, vomitus, saliva and dental plaque, and in various other samples, e.g. suspected non-human reservoirs such as water sources, other putative hosts and food products but also in experimental models such as animal models. However, the PCR based methods are to our knowledge used only for research and not for diagnostics. Some of the reported PCR

methods, with advantages and drawbacks, are summarized and compared with culture and microscopy in Table 1.

Table 1.

	Sensitivity	*Specificity for H. pylori*	*Quantitative assay*	*Time requirement*	*Comments*
Culture	Very low	High	Possible	5-7 days	Detects only live bacteria; does not detect coccoids
Microscopy	Low	High*	Possible	1-2 days*	Inexpensive, detects coccoids; detects dead bacteria
Conventional PCR	High	High	No	1 day	Simple, inexpensive, detects coccoids; detects dead bacteria
Nested PCR	Very high	High	No	1-2 days	Inexpensive, detects coccoids; detects dead bacteria, very high risk of contamination
Real-time PCR	Very high	High	Yes	1 day	Detects coccoids; detects dead bacteria

* When combined with *H. pylori* specific DNA probes or antibodies

Gene expression and regulation

The gene expression and regulation thereof in *H. pylori* have been intensely scrutinized in a number of studies (86), even though *H. pylori* is difficult to manipulate genetically. The early sequencing of two *H. pylori* strains, J99 and 26695 (6); early development of *H. pylori* genome-wide microarrays; and the well known intrinsic genetic variability of *H. pylori* (2) have undoubtedly facilitated the many genetic studies. Among notable findings is the fact that *H. pylori* has remarkably few two-component signaling systems; only 4 have been described to date. The scarcity of the most common regulatory system in prokaryotes indicates that *H. pylori* is highly specialized and has little capacity to adapt to different milieus (86). The relative lack of regulatory switches also suggests that genetic homologies with other bacteria may be misleading in the sense that the genetic regulatory circuits in *H. pylori*, few as they are, may have wider applications than in related bacteria such as *Escherichia coli*.

Mouse and other animal models

The different animal models of *H. pylori* infection that have been developed have different drawbacks and advantages. The mouse model is arguably the most common. It is cheap relative to other mammals and our knowledge of mouse genetics, immunology and physiology is much greater than that of the other animals used to model *H. pylori* infection. Another advantage is that a large number of mouse strains are available, including both inbred

and outbred strains as well as many specific gene knock-out strains. One drawback is however that very few *H. pylori* strains are able to colonize wild-type mice and the most common strain, Sydney Strain 1 (SS1) (90), was passaged more than 20 times to adapt it to the mouse. Another drawback is that the gastric milieu of mice is rather different from that of humans, with higher pH and other naturally occurring bacteria. Furthermore, ulcers and tumors are not developed in *H. pylori* infected mice, although gastritis is developed after 3-4 months of colonization in many mouse strains. Apart from infection with adapted *H. pylori* strains, the mouse is often experimentally colonized with murine helicobacters such as *H. felis* or *H. muridarum*, which mimics some aspects of *H. pylori* infection. One of the earliest animal models used gnotobiotic piglets (80), the main advantage of which is obviously that that they are very similar to humans with respect to genetics, dietary habits and anatomy. However, gnotobotic piglets are difficult and expensive to breed and maintain. Other studies have used guinea pigs (149, 160), which are similar to mice in many respects but their gastric milieu is somewhat more similar to the human. However, rodent-adapted *H. pylori* strains or murine helicobacters are required for infection. Mongolian gerbils (*Meriones unguiculatus*) have been used to study *H. pylori* pathogenesis since gerbils are reported to develop similar pathologies as humans (62, 63, 165, 170), such as ulcers and adenocarcinoma. However, the development of adenocarcinoma in gerbils has been challenged (44). Finally, primates have also been used in some instances, most notably rhesus macaques (*Macaca mulatta*) (43, 47). Primates are closely related with humans and they are naturally infected with *H. pylori*, but they also require highly specialized facilities and staff. However, rhesus macaques were successfully used to model transmission of *H. pylori* (155) and to study global gene expression of *H. pylori* using genome-wide microarrays (65).

Vibrio cholerae and enterotoxigenic *Escherichia coli* (ETEC)

Vibrio cholerae, which causes cholera, and enterotoxigenic *Escherichia coli* (ETEC) are two of the most common etiologic agents of acute infectious diarrhea, together with primarily rotavirus and *Shigella* spp.. Acute infectious diarrhea is a scourge of the developing world, with an estimated 2 to 4 billion cases every year (141) and approximately 2 million deaths. Mortalities caused by diarrheal diseases are especially common among children below the age of 5 years and accounts for approximately 20 % of all childhood deaths which makes it the second most common cause of death after respiratory tract infections in children throughout the world (77). Diarrhea is also very frequent among travelers to developing countries. *V. cholerae* often causes the most severe disease, characterized by a watery stool and massive

loss of fluids, whereas ETEC is believed to cause the largest number of cases annually (141). Apart from *H. pylori* transmission, the focus of this thesis is on possible transmission routes of ETEC and *V. cholerae*, because they are extremely common in Bangladesh, where the field work was carried out. In addition, they are both Gram-negative bacteria just like *H. pylori*, and can thus be studied with identical experimental protocols. Finally, their transmission pathways in Bangladesh and elsewhere are relatively well studied and are thus useful for comparisons to our studies on *H. pylori*.

Epidemiology

The epidemiology of *V. cholerae* and ETEC has been thoroughly studied. The classic studies of cholera transmission in London and elsewhere by John Snow in the middle of the 19th century more or less gave birth to the science of epidemiology. Today, there is ample evidence that both *V. cholerae* and ETEC are transmitted through contaminated water and food (128, 136). *V. cholerae* is probably an environmental species with brackish and sea water as its natural habitat (136) but it also survives in fresh water. Not surprisingly, vibrios including *V. cholerae* can easily be isolated from temperate to tropical marine and estuarine waters. ETEC along with other *E. coli* are primarily enteric bacteria with their natural niche being the gastrointestinal tract but they survive for long periods in water. Both species are common contaminants of drinking water in endemic countries. In Bangladesh, ETEC and *V. cholerae* disease has a typical biannual periodicity (128), with peaks between mid March and June and between mid August and October although they are endemic throughout the year.

In addition to ETEC, at least 4 more groups of diarrheagenic *E. coli* can be distinguished: enteroaggregative (EAggEC), enterohemorrhagic (EHEC), enteroinvasive (EIEC) and enteropathogenic (EPEC). Each group has its own clinical manifestation and epidemiology. In addition, *Shigella* spp. are in fact a group of *E. coli*, very closely related to EIEC, rather than a distinct species even though the historical name has been retained. However, the properties of all pathogenic *E. coli* other than ETEC are beyond the scope of this thesis. There are over 100 O serogroups of ETEC and quite a few H types as well (128); many of the serotypes are also shared by other *E. coli*. Although ETEC disease usually but not always is milder than that of *V. cholerae*, the number of cases is greater with up to 1 billion cases per year resulting in between 300 000 and 500 000 deaths annually, with a majority in young children.

V. cholerae causes pandemic outbreaks of cholera that continue for years or decades. The ongoing seventh pandemic started in the Indonesia in 1961 and rapidly spread to Africa and Latin America. More than 200 O serogroups of *V. cholerae* have been described, but only O1

and O139 are known to cause epidemics of cholera. O1 serogroup is divided in two serotypes, Inaba and Ogawa, which can belong to either of the two biotypes, classical or El Tor. O1 El Tor is the cause of the current pandemic, whereas O1 classical is thought to have caused the previous pandemics. O139 was firstly described in 1993 in the Bengal Bay area where it is still prevalent. It shares certain features with O1 El Tor, described below. The full extent of the current pandemic is difficult to determine, but it has been estimated to be between 3 and 5 million cases each year resulting in 120 000 to 200 000 deaths (172), although these numbers are probably underestimated.

Microbiology

V. cholerae and ETEC belong to the medically important *Vibrionaceae* and *Enterobacteriaceae* families, respectively, of the gammaproteobacteria class of Gram-negative bacteria. *V. parahemolyticus*, *V. vulnificus* and *Aeromonas caviae* are examples of pathogenic *Vibrionaceae* while *Salmonella* spp. and *Yersinia* spp. belong to *Enterobacteraceae*. Both families consist of fermentative, catalase positive, facultative anaerobes with rod-shaped morphology. ETEC are oxidase negative and have peritrichous flagella and *V. cholerae* are oxidase positive and have a single polar flagellum. *V. cholerae* can not ferment lactose, whereas ETEC along with most other *E. coli* can. Both species can grow in low-nutrient media and are able to grow or at least survive in a large temperature range. *V. cholerae* is able to enter a viable but not culturable (VBNC) form under unfavorable conditions (136) and recent research shows that also ETEC has this ability (Åsa Lothigius, personal communication).

Pathogenicity and virulence factors

The clinical symptoms of ETEC and *V. cholerae* are caused by production of enterotoxins at the site of colonization in the upper parts of the small intestine. *V. cholerae* produces and secretes the cholera toxin (CT), a highly potent protein consisting of two structural subunits, a single copy of the active CTA and the pentameric CTB which binds to the intestinal epithelial cells. CT is encoded in the operon *ctxAB* which is part of a lysogenic filamentous bacteriophage (CTXΦ). Many ETEC strains produce a heat-labile toxin (LT) which is a very close structural and functional homologue of CT, with the same arrangement of a single active LTA monomer and the cell surface-binding homopentamer LTB. LT is encoded by the *eltAB* operon which is located in a plasmid. Upon secretion in the small intestine, LT and CT have very similar modes of action. Briefly, the B subunit binds to the eukaryotic

monosialoganglioside cell surface receptor GM_1. The A subunit is then transported into the epithelial cell where it leads to activation of adenylate cyclase followed by increase of cyclic AMP and subsequently loss of water and electrolytes.

The heat stable toxin (ST) that is produced by many ETEC strains is profoundly different from CT and LT in structure and mode of action but the clinical outcome is very similar. There are two types of ST in strains that infect humans, STh and STp, named after their initial discovery in humans and pigs. STh and STp are encoded by the plasmidic genes *estA* and *estB* (other names are sometimes used), respectively, and the two toxins very closely related and have identical function. Briefly, ST is believed to activate guanylate cyclase C, which leads to increase of cyclic GMP and eventually loss of water and electrolytes in a similar fashion to CT and LT. ETEC strains may carry the genes for and produce any combination of LT, STh and STp, although strains producing ST toxin are often associated with more severe disease (128).

In addition to their potent enterotoxins, both *V. cholerae* and ETEC possess several other important virulence and colonization factors. The most important of these will be described briefly. A very important virulence factor of *V. cholerae* is the toxin co-regulated pilus (TCP), which is the receptor for the CTXΦ bacteriophage. TCP consists of approximately 1000 single copies of the TcpA protein, encoded by the *tcpA* gene. O139 and O1 El Tor strains have homologous *tcpA* sequences, whereas O1 classical strains have a significantly different sequence, which makes *tcpA* an interesting target for molecular identification.

ETEC expresses one or more colonization factors, which are important for the pathogenicity. More than 25 colonization factors have been identified in different ETEC strains and the majority of them are fimbrial or fibrillar proteins, with a few exceptions. Although certain patterns of enterotoxin genes, colonization factors and serotypes seem to be common (128), different strains carry different colonization factors and they are thus of little use for rapid and comprehensive identification of ETEC.

Diagnostics and identification techniques

Clinical and research laboratories use many different methods to identify *V. cholerae* in clinical and environmental samples. Several media have been developed for *V. cholerae* isolation and the most common are probably taurocholate-tellurite-gelatin agar (TTGA) or thiosulfate citrate bile sucrose (TCBS) agar plates. To our knowledge the former medium is mostly used for clinical samples and the latter for environmental isolation. Following culture, suspected *V. cholerae* colonies are often confirmed using agglutination, serotyping and other

phenotypic assays. However, the first test during epidemic outbreaks is dark-field microscopic examination directly on fresh stool, where rapidly moving comma-shaped bacilli is a sign of *V. cholerae* infection. For further characterization of isolated strains, both genotypic and phenotypic assays are common, including PCR, pulsed field gel electrophoresis, ELISA and Western blot. Finally, genotypic methods such as PCR are often used to detect VBNC forms of *V. cholerae* in environmental samples.

ETEC is more difficult to identify than *V. cholerae*. It is especially cumbersome to differentiate it from other pathogenic and commensal *E. coli*, which is currently not possible using only culture. For instance, serotyping is not reliable since many of the serotypes are shared by other *E. coli*. ETEC identification instead requires phenotypic or genotypic detection of enterotoxins in a subset of lactose fermenting colonies identified on MacConkey or other selective and differentiating agar. Both PCR and ELISA tests (16, 96, 153) are commonly used, although it should be noted that the phenotypic assays can not distinguish STh and STp. The genotypic assays on the other hand do not reveal if the enterotoxin is expressed, although many PCR positive isolates are reported to express their toxins (152).

Real-time PCR

Polymerase chain reaction (PCR) (117, 140) is a method for exponential amplification of dioxyribonucleotide acid (DNA) sequences which was developed in the late 1980s. In a cyclic process, it amplifies specific DNA sequences with the use of two template DNA binding primer oligonucleotides and a DNA polymerase. PCR is used in a majority of biological and biomedical laboratories for production or analysis of specific DNA sequences. As with many analytical methods, it was evident early on in the use of PCR that a quantitative assay was desirable to provide more information than merely the presence or lack thereof of a certain nucleotide sequence in a biological specimen. Real-time PCR (61) is a quantitative PCR assay in which the amount of nucleotide fragments in the reaction mixture is measured in real-time, i.e. during each cycle of amplification. Arguably, the two most common applications of real-time PCR are gene expression analysis and quantification of pathogenic microorganisms.

Principles

Spectrophotometric determination of fluorescence in the PCR reaction mixture forms the basis of real-time PCR. Two similar but different techniques are used to label the DNA in the reaction mixture, based on fluorescently labeled nucleotide probes and fluorescent dyes, respectively. The fluorescently labeled probes bind specifically to target DNA sequences, i.e.

sequences between the two PCR primers. During the PCR reaction, the probes are incorporated into the amplified copies of the template sequence, which either causes them to emit light of a defined wavelength or to quench previous emission. The resulting changes in emission are then measured by the instrument. Fluorescent dyes, the most common of which is SYBR Green 1 (143), bind to double stranded DNA regardless of sequence. Binding then causes them to emit light which is measured by the instrument. The emitted fluorescence in each reaction is then plotted against the number of amplification cycles. The change in fluorescence becomes significantly different to the background fluorescence after a certain number of cycles, which depends directly on the initial concentration of target sequence in the reaction. The cycle number is determined either visually by the instrument operator or mathematically by the instrument and is referred to as the threshold cycle (C_T) or sometimes as the crossing point (C_P). C_T is used in this thesis. Since the threshold cycle is directly dependent on the number of DNA copies in the reaction it can be used to quantify the initial concentration of the target sequence.

Absolute and relative quantification

Real-time PCR can be used to determine the quantity of a given DNA sequence either in absolute numbers or relative to some other DNA sequence. In absolute quantification, the copy number of the sequence is determined using a serial dilution with known concentration of an identical sequence as a standard curve. The standard curves should ideally span a large concentration range to ensure that the target nucleotide concentration in any investigated sample falls within the range. The standards are often themselves made of PCR product, although entire genomes or gene fragments cloned into bacterial plasmids are also common. Absolute quantification is probably most frequently used to quantify pathogens, which can be done in a variety of different samples.

In relative quantification, the exact copy number of the target sequence is never determined. Instead, the difference in C_T of the target sequence relative to a control sequence amplified in parallel is determined, which may then be transformed to fold change of copy number (127). If the concentration of the control sequence is assumed or determined to be constant in all analyzed samples, the strategy allows comparison of the fold change between the different samples. Relative quantification is mostly used in gene expression analyses. In order to analyze the levels of transcribed mRNAs, reverse transcription of RNA to cDNA is required (164). The efficiency of the reverse transcription assay is almost always assumed to be constant in all samples. The levels or fold change of a target gene cDNA is then estimated

relative to an endogenous control cDNA with the strategy outlined above. One or several so called housekeeping genes - genes with an assumed constitutive and constant level of transcription - are used as the endogenous control cDNAs. In this way, the expression of genes can be compared between individuals or groups; experimental set-ups or treatments; time points in an experiment or treatment; or between organs within an individual.

Real-time PCR in microbiology

As mentioned above, real-time PCR has been used to enumerate bacterial genes or genomes in clinical or other samples. The method has several advantages (see also Table 1). It is highly sensitive and can be targeted against species or strain specific genes or parts of genes although adequate controls against false negative and positive results are very important. The method does however not discriminate between live and dead bacteria and it also detects VBNC forms if they are present in a sample, although the latter could be advantageous in some cases. Real-time PCR may also be used for bacterial gene expression analysis, but there are a few peculiarities of bacterial gene expression which makes it a little more troublesome to analyze than eukaryotic gene expression. For instance, bacterial genes do not contain introns, which increases the risk for amplification of genomic DNA since the cDNA will be identical to the genomic DNA. Furthermore, there are arguably no housekeeping genes in bacteria, since the entire metabolism is regulated in response to external conditions and signals such as quorum sensing. The mRNA half-life of bacteria is also extremely short, in many cases only a few minutes (146), which means that immediate and efficient stabilization of each sample is paramount.

Aims

The overall aims of this thesis were to investigate possible transmission pathways of *H. pylori*, enterotoxigenic *Escherichia coli* (ETEC) and *V. cholerae* in Bangladesh and analyze *H. pylori* virulence gene expression in vivo and in vitro. The specific aims were:

- To develop sensitive and specific quantitative real-time PCR assays targeting conserved sequences in *H. pylori*, ETEC and *V. cholerae.*

- To investigate the risk of waterborne transmission of *H. pylori* and ETEC in a high endemic area in Bangladesh.

- To investigate possible *H. pylori* and *V. cholerae* dissemination through contaminated vomitus and diarrheal stool during outbreaks of diarrheal diseases in Bangladesh.

- To study *H. pylori* colonization dynamics and expression of key virulence genes in an experimental mouse model.

- To compare the expression of key *H. pylori* virulence genes in antral and duodenal biopsies and in experimentally infected animals.

Material and methods

The main methods that were used in the different studies of this thesis are described below.

Bacterial strains and culture conditions

Aerobic enterobacteria (Paper I-III) used for primer specificity tests were grown aerobically overnight at 37 °C on blood or Luria Bertani (LB) agar plates and then inoculated into 10 ml of LB broth and cultured in a shaking incubator overnight at 37 °C and 150 rpm. *Helicobacter* spp. and *Campylobacter* spp. used for primer specificity tests were grown on Columbia agar supplemented with 1 % IsoVitaleX TM for 3 days in microaerobic conditions (5 % O_2, 10 % CO_2, 85 % N_2).

To obtain a high proportion of spiral *H. pylori* for in vitro studies or mouse infection, selected *H. pylori* strains were further grown in 25 ml Brucella Broth supplemented with 5 % fetal calf serum, 10 μg/ml Vancomycin, 5 μg/ml Trimethoprim and 20 U/ml Polymyxin B in sterile 250 ml flasks for 20 h at 37 °C with shaking at 150 rpm under microaerophilic conditions.

Sample collection and treatment

Water samples

In order to study the prevalence of *H. pylori* and ETEC in water in Dhaka, samples of drinking, environmental and waste water were collected.

The municipal water in Dhaka is chlorinated prior to distribution in pipelines to central water pumps, which are open once or twice a day, where community members collect water. The drinking water is then typically stored inside households in jars or open wells up to 24 h and in water tanks or jars on the roof tops up to a few days. Drinking water samples were collected from jars, wells, water pumps and water tanks in the Mirpur area of Dhaka between October 2005 and April 2006.

In addition, environmental (ponds and lakes) and waste water samples from open sewers close to homes and public toilets were collected in Mirpur and other areas in Dhaka between November 2005 and March 2006. All water samples were collected in sterile flasks and transported on wet ice to the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR, B). Drinking water samples were directly filtered onto 0.22 μm Millipore filters. Lake and pond water samples were filtered first through a Whatman filter to remove large particles and then through 0.22 μm filters. One half of the 0.22 μm filters were

used for culturing of *E. coli* on MacConkey agar. Colonies were then tested for ETEC enterotoxin production with GM1-ELISA (16, 153). The second half of each filter was used for DNA extraction and analysis. Waste water samples were centrifuged first at 500 g for 10 min to remove large particles and the supernatant was then centrifuged at 25000 g for 10 min to collect bacteria. Natural drinking water biofilm samples were collected by submerging ethanol disinfected glass slides in water tanks and jars in households in Mirpur. The glass slides were collected after 14 - 30 days and transported on ice to ICDDR, B, where the biofilm was scraped off using the blunt end of a sterile plastic pipette tip. All filters, pellets and biofilm samples were stored at -70 °C until DNA extraction.

Clinical samples

Vomitus or diarrheal stool samples were collected from patients admitted to the ICDDR,B hospital in Dhaka, Bangladesh, for acute diarrheal disease in September 2007 during the second yearly epidemical peak of diarrheal diseases caused by either *V. cholerae* or ETEC or both (37). Samples in volumes between 20 and 300 ml were collected in sterile flasks and transported to the ICDDR,B laboratory. The sample treatment is summarized in Figure 3. Each sample was directly analyzed by dark-field microscopy for presence of vibrios and cultured for ETEC, *V. cholerae*, *Salmonella* spp. and *Shigella* spp. on MacConkey agar, TTG agar and SS agar, respectively. The samples were then centrifuged at 200 rpm for 1 min to pellet large particles and the supernatants were then aliquotted in 1 ml volumes in microcentrifuge tubes. Aliquots for subsequent DNA or RNA analyses were centrifuged for 12 min at 16000 g and pellets were stored at -70 °C until extraction of nucleic acids. One aliquot was immediately used for quantitative culturing of *H. pylori*, *V. cholerae* and ETEC on specific agar plates (Figure 3). Presence of ETEC on MacConkey agar plates was tested by enterotoxin gene multiplex PCR on 5-10 lactose fermenting colonies from each plate. Studies on bacteria in vomitus and stool do not require ethical permits.

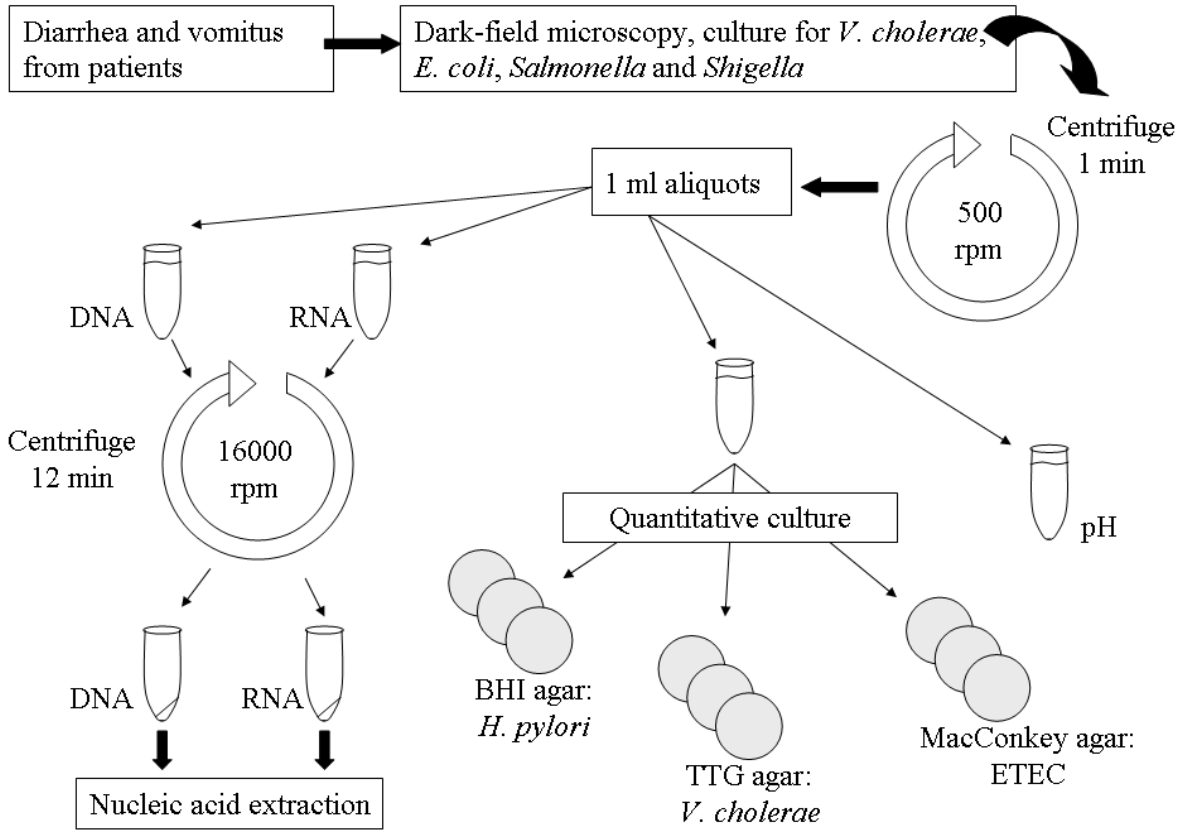


Figure 3: Schematic picture of the treatment of vomitus and diarrheal stool samples after arrival at ICDDR, B laboratory.

In addition to the samples from patients with gastroenteritis, antral and duodenal biopsies were obtained from *H. pylori* seropositive adult individuals with duodenal ulcer and from asymptomatic *H. pylori* seropositive adult individuals visiting the Dhaka Medical College Hospital for endoscopy. Each biopsy was immediately stabilized in RNALater (Qiagen, Hilden, Germany), cut into 2 or 3 pieces and stored at -70 °C. This study was approved by the Research Review and the Ethical Review Committees of ICDDR,B, Dhaka, Bangladesh and the Ethical Committee for Human Research, University of Gothenburg. Informed consent was obtained from each volunteer before participation.

Mouse infection

Infection

Specific pathogen-free (SPF) C57BL/6 mice (Harlan, Netherlands) were housed in microisolators at the Laboratory for Experimental Biomedicine, University of Gothenburg, Sweden, during the study. *H. pylori* strain SS1 (90) was cultured in Brucella broth

supplemented with calf serum and antibiotics under microaerophilic conditions at 37 °C for 24 h as described (131). Female six- to eight-week-old mice were then orally infected by gavage under anesthesia (Isoflurane; Abbott Scandinavia Ab, Solna, Sweden) with approximately 3 x 10^8 CFU of *H. pylori* SS1 suspended in Brucella broth. All experiments were approved by the Ethical Committee for Laboratory Animals in Gothenburg (ethical permit 353/05).

Sample collection

Seven to eight mice were killed at each time-point. Blood was collected under anesthesia from the axillary plexus immediately before the mice were killed and used to prepare serum. The stomachs were opened, stomach contents were removed and the pH of the mucosa was measured by pressing strips of pH papers (Merck KGaA, Darmstadt, Germany) onto the stomach lining. The stomach was washed gently with PBS and the mucosal layer was removed using a glass slide as described (21) and put into 1 ml of Brucella Broth and vortexed briefly. The suspension was used for RNA and DNA extraction and quantitative culture. Volumes of 500 µl, immediately stabilized in 1 ml RNAprotect Bacteria (Qiagen), for RNA extraction and 200 µl for DNA extraction were centrifuged for 10 min at 5800 g and pellets were stored at -70 °C until nucleic acid extraction.

Determination of infection status

Quantitative culture with up to four dilutions of the stomach mucosal layer was performed on blood skirrow agar plates for 7 days under microaeophilic conditions. Serum IgG antibody titers were determined by enzyme-linked immunosorbent assay using a membrane protein preparation of *H. pylori* SS1 for coating (103, 132).

Extraction of nucleic acids

DNA

DNA was extracted from all samples except waste water using the DNeasy Tissue Kit (Qiagen GmbH, Helden, Germany) and eluted in 100-200 µl elution buffer (buffer AE). DNA in waste water samples was extracted with the QIAamp DNA Stool kit (Qiagen) and eluted in 200 µl elution buffer (buffer AE). DNA was kept at 4 °C for short-term or at -20 °C for long-term storage. All DNA extractions were performed in a UV-hood to avoid DNA contamination. Eight to ten samples were processed simultaneously, and one empty

microcentifuge tube was included in each run as an additional negative DNA control to control for possible template contamination.

RNA

For RNA extraction, samples were immediately stabilized in lysis buffer (100 µl TE-buffer with 400 µg ml-1 lysozyme and 350 µl Qiagen Buffer RLT), RNALater or RNAprotect Bacteria reagents (both from Qiagen) and stored at -70 °C. From most sample types, RNA was purified directly from frozen samples using the Qiagen RNeasy Mini kit, eluted in 50 µl RNAse free water and stored at -70 °C. To increase the RNA yield, human tissue specimens, mouse mucosal specimens and some bacterial in vitro cultures (Paper III-IV) were pretreated by two-step bead-milling in a TissueLyser II (Qiagen) before RNA isolation with Qiagen's RNeasy Mini kit as above. All RNA extractions were performed in a UV-hood to avoid contamination with DNA or RNAse.

cDNA synthesis

cDNA synthesis was performed using the Quantitect Reverse Transcription kit (Qiagen). Each sample was DNAse treated (included in the kit) and then divided into two aliquots, one was used to reverse transcribe RNA into cDNA while the other was used as the reverse transcriptase negative control (-RT control) in the subsequent real-time PCR assays. The *Drosophila melanogaster* gene *muc14* cloned into a PCRII vector (a kind gift from Dr. Iris Härd, University of Gothenburg) was transcribed using in vitro transcription (Ambion) and used to determine the efficiency of cDNA synthesis. The single stranded *muc14* RNA was included at a concentration of 10000 copies in each cDNA synthesis performed. The efficiency of cDNA synthesis was then determined for each reaction by correlating the numbers of the exogenous muc14 cDNA to a cDNA preparation of pure *muc14* RNA. cDNA was stored at -20°C.

Real-time PCR

Primer design

New real-time PCR primers targeting selected virulence genes in *H. pylori*, ETEC and *V. cholerae* were designed to optimize real-time PCR performance (Table 2). Two to five sequences of each target gene were derived from the NCBI nucleotide database (www.ncbi.nlm.nih.gov) and global alignments were performed with the Web-based EBI alignment tool EMBOSS (www.ebi.ac.uk/Tools/emboss/align/) (137). Conserved regions

were identified manually in the aligned sequences and primer pairs targeting the conserved regions were designed using Primer Express software (Applied Biosystems, Foster City, CA, USA), with default settings (Table 2). Primer sequences were analysed in a BLAST search (NCBI genome database) against all available genomes to confirm primer pair specificity.

Table 2.

Species	Target gene	Direction	Name	Sequence 5'=>3'
H. pylori	*cagA*	Forward	CagA-rt-F1	TGATGGCGTGATGTTTGTTGA
H. pylori	*cagA*	Reverse	CagA-rt-R1	TCTTGGAGGCGTTGGTGTATT
H. pylori	*flaA*	Forward	FlaA-rt-F1	ATGACGGTGGCGGATTCTT
H. pylori	*flaA*	Reverse	FlaA-rt-R1	GATAATCCCCATGCCGTCATT
H. pylori	*glmM*	Forward	GlmM-rt-F1	GCTCACTAAAGCGTTTTCTACCATATAG
H. pylori	*glmM*	Reverse	GlmM-rt-R1	ATTGCTGCCGGATTGTATTTTAA
H. pylori	*hpaA*	Forward	HpaA-rt-F1	ACTTTCTCGCTAGCTGGATGGTA
H. pylori	*hpaA*	Reverse	HpaA-rt-R1	GCGAGCGTGGTGGCTTT
H. pylori	*ureA*	Forward	UreA-rt-F1	TATGGAAGAAGCGAGAGCTGGTA
H. pylori	*ureA*	Reverse	UreA-rt-R1	GAGTGCGCCCTTCTTGCAT
H. pylori	*vacA*	Forward	VacA-rt-F1	CGCTATCAATCAGCATGATTTTG
H. pylori	*vacA*	Reverse	VacA-rt-R1	CCCGCATCATGGCTATCAAT
V. cholerae	*tcpA*	Forward	TcpAET-rt-F1[a]	GGTCAGCCTTGGTAAGGTTTCA
V. cholerae	*tcpA*	Reverse	TcpAET-rt-R1[a]	AAATCCCCATAGCTGTACCAGTGA
V. cholerae	*ctxB*	Forward	CTB-rt-F1	AGCGATTGAAAGGATGAAGGATAC
V. cholerae	*ctxB*	Reverse	CTB-rt-R1	CACATAACTTTTCGACTTTAGCTTCAGT
ETEC	*estA*	Forward	STh-rtF1	AAGTGGTCCTGAAAGCATGAATAGTAG
ETEC	*estA*	Reverse	STh-rtR1	ACCCGGTACAAGCAGGATTACA
ETEC	*estB*	Forward	STp-rtF1	GCAAAATCCGTTTAACTAATCTCAAA
ETEC	*estB*	Reverse	STp rtR1	AATTGCCAACATTAGCTTTTTCATG
ETEC	*eltB*	Forward	LT-RTf	GGCAGGCAAAAGAGAAATGG
ETEC	*eltB*	Reverse	LT-RTr	TCCTTCATCCTTTCAATGGCT

[a] Specific for El Tor *tcpA*

Standard curves

To facilitate absolute quantification of bacterial genomes and cDNA, calibrated standard curves with known concentration of bacterial genomes or PCR products from relevant bacterial genes were made. Standard curves for *H. pylori* and *V. cholerae* were made from RNAse-treated genomic DNA extracted from whole-genome sequenced *H. pylori* strain J99 (6) and *V. cholerae* El Tor strain N16961 (60). Since the enterotoxin genes for ETEC (*estA*,

estB and *eltB*) are encoded in plasmids and copy number per genome may vary from cell to cell, two separate sets of standard curves were made for ETEC; using either RNAse-treated genomic ETEC DNA or PCR products of each enterotoxin gene. Three enterotoxin stable ETEC reference strains, ST64111 (STh), Takeda 101 (STp) and 286 C2 (LT) were selected for use in genomic DNA standard curves. Concentration of extracted genomic DNA or amplified gene sequences was determined by measuring absorption at 260 nm in a spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and the molar concentration was calculated. The standard curves were made by tenfold serial dilutions in elution buffer (Qiagen) from 10^6 down to 10 copies per µl to cover a large range of possible target concentration in the samples.

Real-time PCR

Real time PCR analyses were performed on an ABI7500 real-time PCR instrument (Applied Biosystems). The reactions were run in 96-well plates (Applied Biosystems) with a total volume of 20 µl in each reaction. The optimized PCR mix contained 10 µl SYBR Green I Real-time PCR Master Mix (Applied Biosystems), 8-10 pmole of each primer, 6-6.4 µl water (nucleotide-, DNAse- and RNAse-free) and 2 µl sample.

No template controls (water) and a standard curve were included in each run. When appropriate, several other negative controls were analyzed to ensure that no contamination either of exogenous DNA in all samples or of endogenous genomic DNA in cDNA preparations had occurred at any point during the sample treatment.

Results and comments

Real-time PCR for quantification of DNA and mRNA of gastrointestinal, gram-negative bacterial pathogens (Paper I-IV)

Many pathogenic bacteria and especially *H. pylori* can be difficult to isolate from disadvantageous environments. Consequently, we developed real-time PCR assays for detection and quantification of *H. pylori*, ETEC and *V. cholerae* to be able to enumerate and characterize these species in a wide range of samples. Real-time PCR is an excellent method for this purpose since it is highly sensitive and specific and can be optimized to study several different bacteria in the same sample. Furthermore, it can be applied to quantify either DNA or RNA, which allows both enumeration of bacterial genes and characterization of gene expression. Throughout the studies, the different real-time PCR methods and applications have been continuously and extensively evaluated with regard to specificity, sensitivity and robustness. These experiments have shown the suitability of the technique to quantify the number of bacteria (Paper I-IV), the number of gene copies per bacterial genome (Paper I) and bacterial gene expression (Paper III-IV). In this part of the thesis, the development of the main real-time PCR applications will be described.

Development of real-time PCR quantification of *H. pylori*, ETEC and *V. cholerae* genomes and gene expression (Paper I-III)

Selection of genes

In order to accurately quantify bacteria with real-time PCR, it is crucial to select target genes that are both specific for the bacterial species and universally conserved within the species. For bacterial pathogens, virulence genes often fulfill these criteria. For ETEC, the enterotoxin genes *estA* (STh toxin), *estB* (STp toxin) and *eltB* (LT toxin B subunit) are obvious target genes (table 2), since they are the genes that confer the enterotoxicity to ETEC strains and separate them from other types of enteropathogenic and commensal *E. coli*. Similarly, for *V. cholerae*, the cholera toxin is the natural target, although it is found in a few more vibrio

serogroups than O1 and O139. In addition, El Tor *tcpA* (toxin co-regulated pilus A) was selected to be able to rapidly distinguish between El Tor and classical strains. For *H. pylori*, literature searches, bioinformatic analyses and previous research suggested that *hpaA* (*Helicobacter pylori* adhesin A) and *glmM* (phosphoglucosamine mutase) would be suitable target genes. Previous similar studies and assay development on *H. pylori* have used *hpaA*, *glmM*, *ureA*, 16S rRNA and other genes (24, 28, 45, 50, 59, 64, 66, 67, 107, 130, 147, 168). In most of these cases the genes or targeted regions within the genes are specific for *H. pylori* but sometimes for *Helicobacter* spp..

Primer design and specificity tests

Sequences for the above genes from several different strains of each species were linearly aligned to find conserved regions. Primers annealing to the conserved regions were then designed using Applied Biosystems' Primer Express software. The specificity of each primer pair was tested in silico, using the nucleotide blast algorithm at the NCBI homepage (blast.ncbi.nlm.nih.gov/Blast.cgi), showing that each pair was specific for the gene. Primer pairs were also tested against a genomic DNA library of different gastrointestinal bacteria (Table 1 in Paper I, Table 1 in Paper II), again showing that the primers are specific.

Standard curves and sensitivity tests

Using serial dilutions of genomic DNA from reference strains, *V. cholerae* N16961, *H. pylori* J99, and ETEC ST64111 (STh), Takeda 101 (STp) and 286 C2 (LT), the sensitivity of the real-time PCR reactions was determined to be 2-3 genomes per reaction (Figure 4A; Paper I, Paper II). The serial dilutions were then used as standard curves to quantify the bacterial genome numbers in subsequent studies. To be able to prepare high purity DNA suitable for real-time PCR, we chose the DNeasy Blood and Tissue mini kit from Qiagen, which is ubiquitously used in molecular biology laboratories. We evaluated the DNeasy kit using diluted *H. pylori* and ETEC and found that the kit was able to extract DNA in a linear fashion from bacterial numbers ranging from 100 up to at least 10^8 per extraction column (Figure 4B). The assays were then used in combination with the DNeasy kit throughout our studies, with the exception of a few cases where the very similar QIAamp DNA Stool mini kit was used.

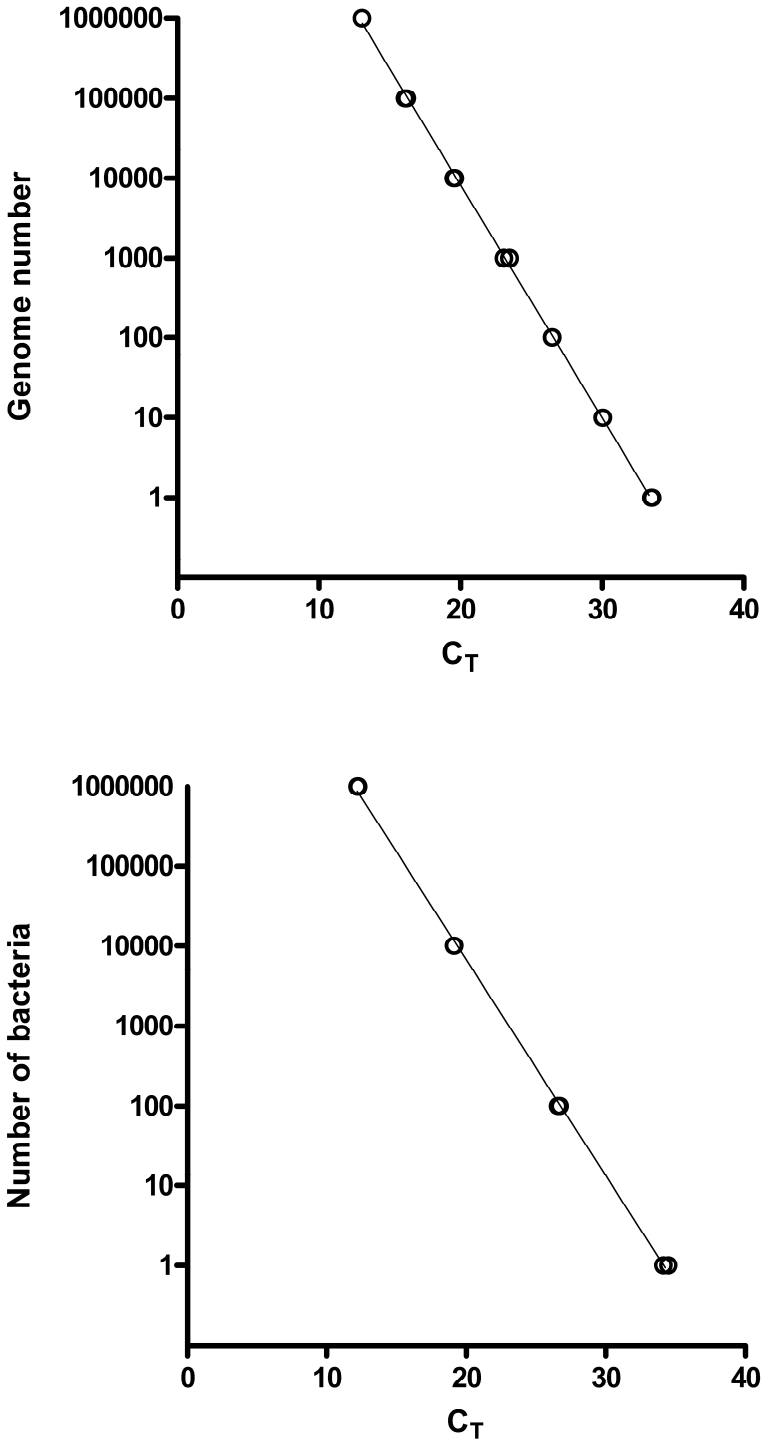


Figure 4: Data from the initial evaluation of real-time PCR and DNA extraction procedures.
A. A representative DNA standard curve made from serial tenfold dilutions of purified *H. pylori* strain J99 DNA. Genome number per µl plotted against C_T.
B. Real-time PCR analysis of DNA extracted from hundredfold serial dilutions of *H. pylori* strain Hel703. DNA from each dilution was eluted into 100 µl buffer. The corresponding cell number per µl eluted DNA is plotted against C_T.

Determination of enterotoxin gene copy numbers of different ETEC strains (Paper I)

Quantitative PCR may be used to determine different numbers of gene copies, such as for instance allelic analyses. Following up on our initial results to quantify ETEC genomes in various samples, we wanted to see if the established real-time PCR assays could be used to estimate the copy number of ETEC toxin genes in different strains. Since the toxin genes are carried on plasmids, their number may vary between strains and also under different environmental conditions. We quantified both the number of genomes with a genomic DNA standard curve and the number of each gene with a PCR product standard curve. Using this approach, we could successfully estimate the gene copy number in 35 ETEC strains producing different combinations of enterotoxins, as determined by GM1-ELISA analyses. However, in this experiment only a single environmental condition (4 h culture in LB broth at 37 °C and at 150 rpm) was used and it thus remains to be seen if the copy number is influenced by e.g. growth conditions.

Development of a method to quantify absolute numbers of bacterial mRNA (Paper III-IV)

In addition to enumerating *H. pylori*, ETEC and *V. cholerae*, we also wanted to characterize the virulence gene expression of the bacteria and compare the expression between different sample types, such as environmental samples, in vitro cultured bacteria and in vivo samples from humans or animal models. Early on in the method development, rather than relying on housekeeping genes for normalization between samples, we decided to use absolute quantification of reverse transcribed mRNA copies. Absolute quantification of mRNA can be done using either of two standard curves, one based on DNA (such as genomic DNA or PCR products) or one based on RNA (serially diluted and then reverse transcribed). Briefly, the main advantage of using a DNA-based standard curve is that it is very stable and the main drawback is that it does not take the reverse transcription (RT) into account, i.e. it quantifies the number of cDNA copies of the target mRNA sequence rather than the number of mRNA transcripts. Conversely, a standard curve made from purified mRNA:s does take the RT efficiency into account, but instead it assumes that all samples have the same RT efficiency as the purified mRNA used for the standard curve, which is rarely true and especially not in complex samples. The RT efficiency varies greatly between different samples, depending on mainly nucleotide concentration and reverse transcriptase inhibition. In our case, the

efficiency of reverse transcription reactions had to be taken into account, since it could not be assumed to be identical in the highly diverse sample types that we were interested in. Instead, we chose to estimate the RNA efficiency in each analyzed sample by spiking them with a known amount of an in vitro transcribed *Drosophila melanogaster* gene (*muc14*), which has no homology to any known prokaryotic, human or murine gene. For each sample, the threshold cycle of this synthetic mRNA was then compared to the threshold cycle of a reverse transcribed pure sample of the same amount of this gene. The RT efficiency for each sample could then be calculated. Finally, assuming that the RT efficiency is the same for all transcripts in each sample, we could quantify our genes of interest using a genomic DNA standard curve. For each sample, after quantifying the number of genomes, transcript numbers were normalized for each gene by dividing the number of transcripts with the number of genomes, which allowed us to compare the virulence gene expression between different samples.

Dissemination of *H. pylori*, ETEC and *V. cholerae* (Paper I-III)

The main objective of these studies was to evaluate different previously suggested putative transmission pathways of *H. pylori*, ETEC and *V. cholerae* in Bangladesh, an area where all three pathogens are endemic. However, our focus has been on *H. pylori*. Since many bacteria, may be difficult to culture from environments that are unfavorable to them, we adopted real-time PCR as a complement to culture. Alternative quantitative methods are highly valuable especially for studies of *H. pylori*, which is nearly intractable to culture from environmental samples. Firstly, we investigated the possibility that ETEC and *H. pylori* are waterborne through analysis of drinking, environmental and waste water samples. *V. cholerae* was not included in these studies, since it is already established that it may be waterborne. In a second study, we investigated the presence of *H. pylori* and *V. cholerae* in diarrheal stool and vomitus during outbreaks of diarrheal diseases with the purpose of comparing the number of *V. cholerae* between vomitus and stool and to determine if *H. pylori* may be disseminated through diarrheal stool or vomitus or both. ETEC was not included in the latter study for the simple reason that the patients that we collected material from were suffering mainly from cholera.

Development of filtration and DNA extraction protocols (Paper I and II)

To be able to collect bacteria from water samples, we chose a commercially available standard microbiological filter with 0.22 μm porosity, which allows efficient collection of bacteria from variable volumes of water. Two different approaches to extract DNA from filters were evaluated using cultured bacteria suspended in PBS. We could show that DNA extraction directly from the filter was approximately 1000-fold better than washing the filter with PBS followed by centrifugation and extraction from the pellet (Figure 5). Using filtration followed by DNA extraction with the DNeasy kit, the lower detection limit of the assays was determined to be 250 genomes per sample (or filter) for both ETEC and *H. pylori*. For *H. pylori*, this was then further corroborated by testing the assay sensitivity on serial dilutions of two different strains of *H. pylori* that had been incubated for up to 100 days in tap or sea water from Gothenburg, showing similar results (Paper II: Figure 1).

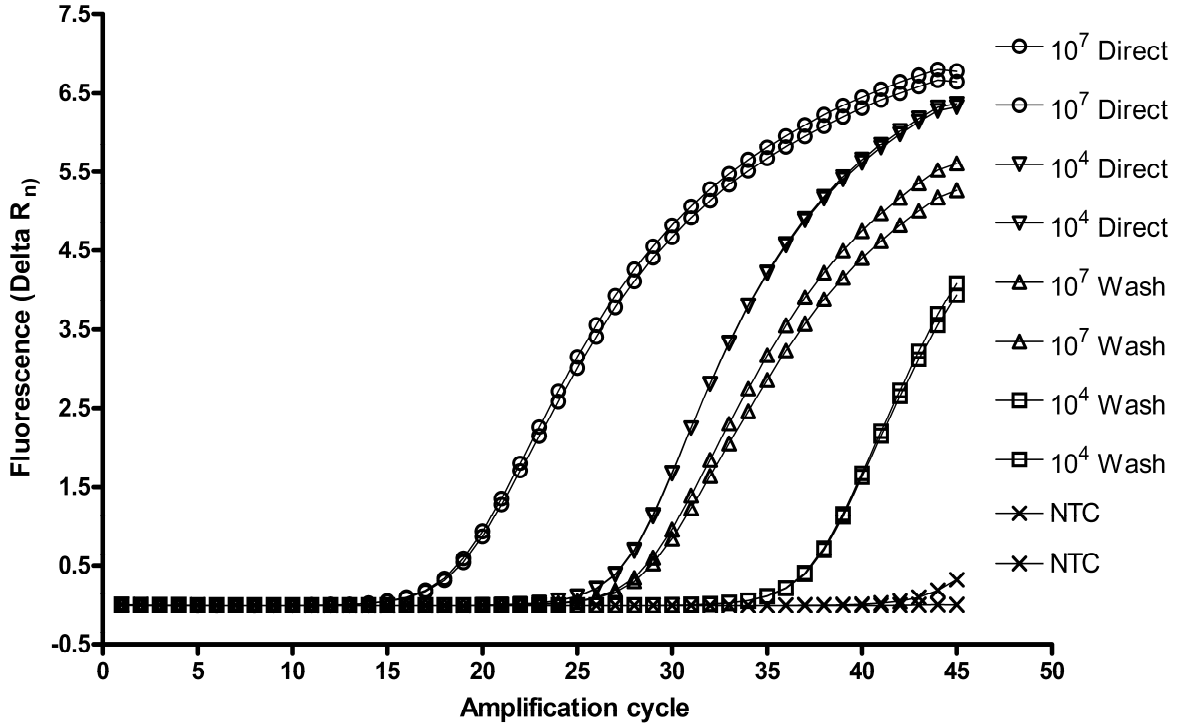


Figure 5: Real time PCR amplification curves showing a comparison of the efficiency of the two different methods for extracting DNA from *H. pylori* suspensions with either 10^7 or 10^4 bacteria per ml collected with microbiological filters. Direct = DNA extraction directly from the filter; Wash = each filter was submerged in PBS to rinse off bacteria and DNA extraction was performed following centrifugation of the PBS. The former method was 1000-fold more sensitive, i.e., the amplification curve reached the threshold detection level approximately ten PCR cycles earlier. NTC = No template control.

Detection of ETEC enterotoxin genes in drinking and environmental water during epidemic and endemic periods in Bangladesh (Paper I)

Using the real-time PCR assays targeting ETEC enterotoxin genes *estA* (STh toxin), *estB* (STp toxin) and *eltB* (LT toxin B subunit) and optimized collection and DNA extraction protocols we first conducted a study to determine the presence and quantity of ETEC in drinking and environmental water samples in Mirpur, a poor slum area in Dhaka. Drinking water samples were collected from jars and buckets in households in the Mirpur area and environmental water was collected from lakes and ponds in the larger Dhaka area. ETEC was found in 18 out of 26 (69 %) of the analyzed drinking water samples and in 8 out of 13 (62%) of the analyzed environmental water samples with real-time PCR and in 2 drinking water and 4 environmental water samples with MacConkey agar culture followed by GM1-ELISA. Of the three different toxin genes, *estA*, encoding STh, was identified in 25 samples, *estB* (STp) in 3 samples and *eltB* (LT) in 18 samples. Although *estA* was the most frequent gene

identified, *eltB* was found in higher numbers, corresponding to a mean number of 166.8 (SEM = 77.87) bacteria per ml sample. In contrast, *estA* levels corresponded to 63.84 (SEM = 22.03) bacteria per ml and *estB* levels to 15.33 (SEM = 6.333) bacteria per ml.

We could also show that drinking water samples positive for ETEC were significantly more frequent (P value = 0.0107) during the epidemic period (October 2005) than in the colder winter season (November - February 2005) when ETEC disease is less common in Dhaka.

Although our results support previous findings that ETEC may be spread through water (16), our study also indicates that ETEC is present in drinking water even during non-epidemic periods. The clinical implications of this finding require more research, it may be that water constitutes a risk for ETEC year-round which is supported by the fact that ETEC is endemic in Bangladesh, but it may also be that ETEC is either less able to survive and persist in water or that it is dormant and possibly less infectious during colder periods and hence results in fewer cases of disease. In any case, the results indicate that the risk of waterborne ETEC transmission is higher during the epidemic periods.

Failure to detect *H. pylori* in drinking, environmental and waste water and drinking water biofilms in Dhaka (Paper II)

In an extended study of water sources in Dhaka, the samples that were analyzed for ETEC and additional samples of drinking, environmental and waste water were analyzed using the two *H. pylori* real-time PCR assays detecting *glmM* and *hpaA*. None of these samples were positive for *H. pylori*. Since PCR assays used in diagnostic or forensic studies have been reported to have a relatively high risk of both false positives and false negatives, we then conducted a number of control experiments to exclude the most common causes of falsely negative results. However, the control experiments showed that the samples were true negatives, with the exception of 10 % of the drinking and environmental water samples that had a very high level of PCR inhibitory substances (Figure 6). It should thus be noted that these 10 % of the samples may be falsely negative results. In addition, the detection level of the assays in another 50 % of these samples was increased from 250 to 2500 genomes per sample since the PCR inhibition in these samples required them to be diluted tenfold. Taken together, our results thus show that levels of *H. pylori* were below at most 2500 genomes per 150 ml in 90 % of the samples; levels in the remaining 10 % of samples could not be determined. Finally, to further confirm the absence of *H. pylori* in drinking water, we also analyzed naturally formed biofilms from drinking water in the households of Mirpur. Glass slides were submerged and allowed to form biofilms for approximately one month. The biofilms were

scraped off the glass slides and analyzed for presence of bacterial DNA. While ETEC was found in the biofilms (unpublished data) the samples were again negative for *H. pylori*. The results taken together lead us to the conclusion that it is highly unlikely that *H. pylori* is predominantly spread by water in this area.

Failure to detect *H. pylori* in environmental, waste and drinking water in Mozambique (unpublished)

Our findings in Bangladesh strongly indicate that *H. pylori* is not waterborne in this particular high endemic area, although it is difficult to extrapolate negative results to other areas. In addition to analyses of samples from Dhaka, we also analyzed samples from a similar but less extensive study in an area in Mozambique with poor sanitary conditions (samples were kindly provided by Malin Lothigius and Johanna Bengtsson, Linköping University, and Bodil Hernroth, Kristineberg Marine Research Station). Forty nine samples of environmental and drinking water and effluents from a waste water treatment plant were collected in Maputo and surrounding areas in April and May 2008. The samples were filtered using the same equipment and protocol as in Dhaka, and DNA was extracted in the lab in Gothenburg using the same protocol as described above. Samples were then analyzed for presence of *H. pylori* using the two real-time PCR assays. Again, all samples were negative for both *H. pylori* genes (unpublished results). Although these samples were not subjected to the same rigorous controls for false negatives, the results still indicate that the risk for waterborne *H. pylori* also in this developing country is low.

Quantification of *H. pylori* and *V. cholerae* in vomitus and diarrheal stool during an epidemic outbreak of gastroenteritis (Paper III)

In Bangladesh, diarrheal diseases are endemic and occur in biannual outbreaks, usually lasting from mid March to early June and from mid August to October, during which not only ETEC and *V. cholerae* disease are occurring, but also new infections of *H. pylori* (17). Since the transmission of *H. pylori* is unlikely to be waterborne in the area, we were intrigued by other previously suggested routes, primarily directly from person to person through either diarrheal stool or vomitus or both. However, considering that *H. pylori* can be difficult to isolate even from its natural niche, we decided to use real-time PCR as a complement to culture. Firstly, the feasibility of quantitative PCR analysis in especially fecal specimens, which has been reported to contain large amounts of PCR inhibitors, had to be determined. Initial tests were consequently carried out on fecal pellets from 17 BALB/c mice (pellets were kindly provided

by Erik Nygren), of which 9 were experimentally infected with *V. cholerae* O139 strain MO10 and 8 infected with *V. cholerae* O1 strain H1. Real-time PCR data and viable counts showed very good agreement (Figure 6).

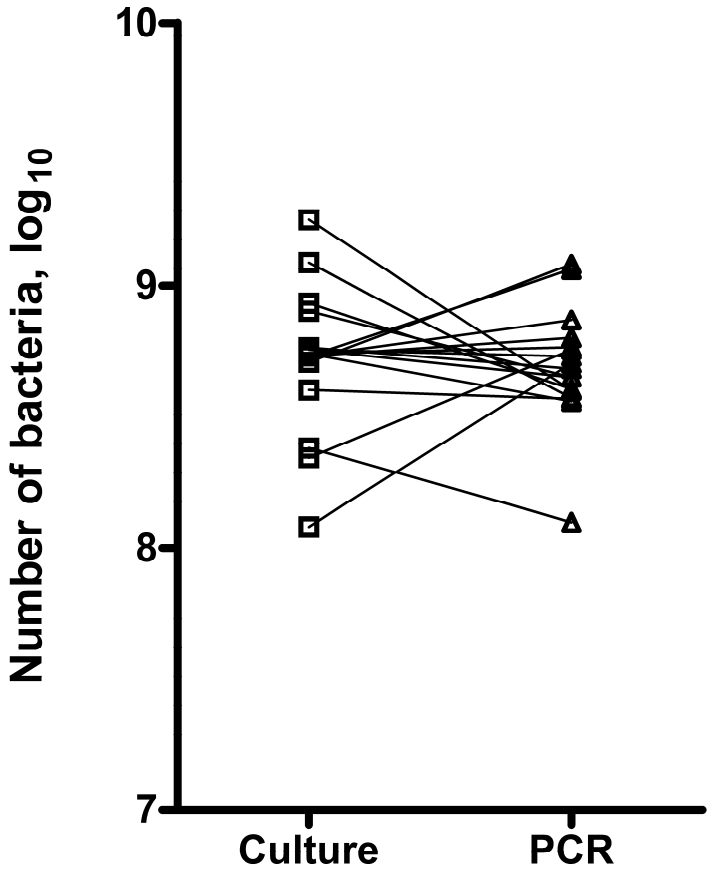


Figure 6: Comparison of quantitative culture and real-time PCR on *V. cholerae* in fecal pellets from mice experimentally infected with *V. cholerae*. For each mouse, 1-2 pellets were used for DNA extraction and real-time PCR, and 2-4 pellets for culture. All data are normalized against total wet fecal weight.

Encouraged by these results, we undertook a clinical study comprising 28 individuals admitted to the ICDDR, B hospital in Dhaka for acute diarrheal disease during September 2007. From the 28 patients, we were able to collect 23 diarrheal stool and 26 vomitus samples, of which 21 were paired samples from the same patient, and analyzed the specimens using standard microbiological plate cultures. However, culture tests are not routinely performed on or optimized for vomitus, and culture from vomitus may accordingly be more uncertain than culture from stool. The culture analyses showed that the likely cause of disease in 23 of the 28 patients was *V. cholerae* infection, with 21 of 23 stool samples (91 %) and 12 of 26 vomitus samples (46 %) culture positive for *V. cholerae*. Apart from evaluating diarrheal stool and

vomitus as possible sources of *H. pylori* infection, the collected material would therefore also allow us to compare *V. cholerae* bacteria shed in stool and vomitus.

All stool and vomitus samples were next analyzed with real-time PCR using *ctxB* and *tcpA* primers for *V. cholerae* and *glmM* and *hpaA* primers for *H. pylori* to enumerate the bacterial genomes. To determine the presence of PCR inhibitors, all samples were also diluted tenfold and analyzed again. In the samples where the genome numbers were not decreased by the dilution factor, which indicates inhibitors, the value obtained from the diluted DNA was multiplied by ten and used for further analyses. No sample was completely inhibited, but 3 out of 23 stool samples (13 %) and 3 out of 26 vomitus samples (12 %) showed some inhibition in the undiluted DNA. By comparing the obtained genome numbers with values from quantitative *V. cholerae* culture, we could show strong correlation between the two methods ($R^2 = 0.69$, P value < 0.0001) (Paper III: Figure 1). Furthermore, the results showed that the numbers of *V. cholerae* were significantly higher ($P < 0.0001$) in stool (median genome number = 2.47×10^6) than in vomitus (median genome number = 2.73×10^4) (Paper III: Figure 2). This result is in agreement with previous reports, suggesting that *V. cholerae* is predominantly shed in feces and transmitted through the fecal-oral route (136). When analyzing the clinical samples for numbers of *H. pylori* bacteria using both quantitative culture and real-time PCR a different distribution was found. Thus, using real-time PCR, we found significantly higher numbers of *H. pylori* ($P < 0.0001$) in vomitus (median genome number = 4.35×10^5 ml^{-1}) than in stool (median genome number = 7.33×10^2 ml^{-1}), indicating that *H. pylori* is more likely to be disseminated through the gastric-oral route than the fecal-oral route during the outbreak. Unfortunately, it was not possible to determine the presence of *H. pylori* in vomitus and stool specimens using culture due to overgrowth of other bacterial and fungal species, despite using medium supplemented with selective antibiotics developed for isolation of *H. pylori* from clinical samples. Ultimately, this means that the real-time PCR method could not be validated for *H. pylori*. It was also not possible to conclude whether the *H. pylori* genomes we found in the clinical specimens were derived from viable or dead bacteria. However, the generally low levels of PCR inhibitors and the high numbers of *H. pylori* in vomitus suggests that it may be possible to use vomitus for non-invasive *H. pylori* diagnostic tests or to test for instance presence of antibiotic resistance genes and the *cag* PAI status.

H. pylori gene expression in different stages of infection in humans, in the mouse model and in in vitro culture (Paper III-IV)

Since we had identified large numbers of *H. pylori* in vomitus and, to a lesser extent, in diarrheal stool, but had failed in our attempts at isolating them, we wanted to use reverse transcriptase real-time PCR to characterize the expression of selected virulence genes in *H. pylori* in the different samples, in the hope of finding evidence of gene transcription. In addition to *V. cholerae* and *H. pylori* in stool and vomitus, we also wanted to investigate *H. pylori* gene expression in human gastric biopsies and in experimental *H. pylori* infection of mice and in in vitro grown *H. pylori*. The reason for these studies was initially to compare findings from vomitus and stool with *H. pylori* in its natural niche. However, we also realized that our approach could be used to characterize the expression of suggested vaccine targets both in infected individuals and in commonly used models of *H. pylori* infection and growth, i.e., the mouse model and in vitro cultures, respectively, with the aim of finding highly expressed target antigens.

Quantification of *H. pylori* in vivo (Paper III-IV)

To be able to normalize gene expression against number of bacteria in different samples for comparison of expression levels, *H. pylori* genomes were firstly quantified in all mouse model samples (Paper IV) and in antral and duodenal biopsies from a group of *H. pylori* positive individuals, comprising both asymptomatic subjects (n = 6) and patients with peptic ulcers (n =6) (Paper III). Comparing the *H. pylori* genome numbers between antral and duodenal biopsies from both symptomatic and asymptomatic individuals, we found significantly higher numbers (P value = 0.0010) in the antrum (Figure 7), which is in agreement with previous reports (57).

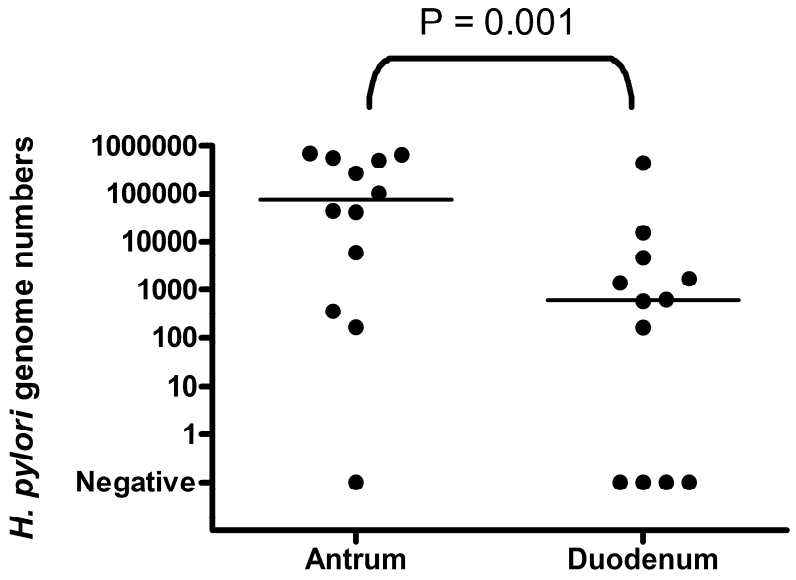


Figure 7: The number of *H. pylori* genomes in antral and duodenal biopsies from asymptomatic subjects and patients with peptic ulcers. Median value for each group is indicated.

In the mouse model, we could show that there was general agreement in gastric mucosa between viable counts and real-time PCR genome numbers (Paper IV: Figure 1 and 2), suggesting that the method is useful for analyses of gastric samples. There are reports of possible PCR inhibition problems in gastric samples, presumably due to the presence of mucins. However, we could not find evidence of significant inhibitory effects either in human or murine samples. The observed lack of inhibition in the samples may be due to either that the DNA extraction method is efficient at removing inhibitors such as mucins, or that these substances were not present in the analyzed biopsies. Regardless of which of the two explanations may be the case, our results suggest that real-time PCR analysis of purified gastric DNA is a promising method for quantifying *H. pylori* in clinical specimens, including for diagnostic purposes. It may be particularly useful to identify falsely negative results in biopsy cultures. Obviously, it could also be used to test for antibiotic resistance genes and *cag* PAI positivity, as suggested for vomitus above.

Transcriptional analysis of *H. pylori* and *V. cholerae* virulence genes in vomitus and diarrheal stool (Paper III)

To determine the virulence gene activity of *H. pylori* in vomitus and stool, we designed primers against key *H. pylori* virulence genes cytotoxin-associated gene A (*cagA*), flagellin A subunit (*flaA*), urease A subunit (*urea*) and the vacuolating cytotoxin (*VacA*). In addition, we were also interested in the *H. pylori* adhesin A (*hpaA*), which has been reported to be essential for colonization of mice (26) and for which we had already designed primers. The gene expression of *V. cholerae* in vomitus and stool has been previously studied using microarrays (88) validated with real-time PCR, and could thus be used to evaluate the feasibility of real-time PCR in the present study by analyses of the expression of cholera toxin (*ctxB*) and toxin co-regulated pilus (*tcpA*), for which we already had primers.

Results from expression analyses in the stool and vomitus samples indicated that both bacterial species express the studied genes to various degrees in the different samples. The results for *V. cholerae* showed that *tcpA* expression was higher in vomitus than in stool and uncoupled from *ctxB* expression, which is in agreement with previous studies (88). It thus seemed reasonable to assume that the method gave reliable estimates of gene transcripts in these sample types. For *H. pylori*, there was a striking difference between vomitus and stool, since a majority of the vomitus samples showed expression of all five analyzed genes whereas the stool samples showed no evidence of expression of the same genes. Although this is probably in part explained by the lower numbers of *H. pylori* in the stool samples, the absence of detectable transcripts even in the stool samples with high numbers of *H. pylori* indicates that gene expression in *H. pylori* is down-regulated in diarrheal stool. Comparing the relative levels of the five *H. pylori* virulence genes in the vomitus samples, a clear pattern emerges (Paper III: Figure 3A). *flaA* and *ureA* are highly expressed, which is expected since the proteins they encode, flagellin and urease, are the two most abundant protein complexes found in *H. pylori*. Furthermore, *cagA* is expressed at a similar level as *flaA* and *ureA*, whereas *hpaA* and *vacA* are expressed at lower levels relative to the other 3 genes. We could not find any significant differences in gene expression between vomitus samples with different pH even though the pH of the samples ranged from approximately pH 1.5 to 7.5. This finding is remarkable in light of the many genes that have been reported to be transcriptionally regulated in response to pH in vitro (109), but it is possible that this regulation is less pronounced in vivo.

As suggested above, our results again suggest that vomitus is an interesting source of *H. pylori* from infected individuals. Vomitus specimens may be obtained without invasive

procedures, and can thus be easily and safely used to collect material for gene expression analyses.

H. pylori virulence gene expression in human gastric biopsies (Paper III)

Our results from analyses of the gene expression in *H. pylori* in vomitus are not very informative in themselves, apart from indicating that the bacteria are transcriptionally active. Accordingly, we wanted to compare the gene expression of *H. pylori* in vomitus and in the human gastric mucosa. It would have been preferable to compare expression between these samples from the same individuals, but it was not possible to obtain stomach biopsies from the cholera patients. Instead we performed the same analyses in antral and duodenal biopsies from a group of *H. pylori* positive individuals, asymptomatic or with peptic ulcers, living in the same geographic area. In the antral biopsies, the relative expression of the five genes (*cagA*, *flaA*, *hpaA*, *ureA* and *vacA*) was similar to that in vomitus but with approximately a hundredfold more transcripts per bacterial genome (Paper III: Figure 4A). It may be noted, however, that *hpaA* is expressed at almost the same level as *cagA*, *flaA* and *ureA* in the antral biopsies, indicating that it may be up-regulated in the antral gastric mucosa. We could not identify significant differences in *H. pylori* gene expression between symptomatic and asymptomatic individuals. Levels of the different genes in human duodenal biopsies were generally approximately tenfold lower than in the antrum and much fewer of the duodenal biopsies showed evidence of *H. pylori* gene expression. The differences in virulence gene transcripts per genome between vomitus and antral and duodenal biopsies may be explained in different ways. The lower transcription per genome found in primarily vomitus and duodenal biopsies may correspond to down-regulation of these specific genes or to systemic down-regulation of all genes in most of the *H. pylori* present in the sample. The differences could also be explained by different proportions of transcriptionally active bacteria in the different samples, i.e., that some of the bacteria in for instance vomitus are dormant or dead. It seems likely that both down-regulation and different bacterial populations may be responsible for the observed differences, although further studies are needed to find out to what extent.

Finally, our studies also suggest that our strategy for studying bacterial virulence gene expression in the infected host is highly promising since it may be used to determine changes in transcript levels between individuals. Previous studies of *H. pylori* gene expression in vivo has been performed with microarrays. Microarrays studies are obviously very valuable since they measure the global transcription, but the technique requires large amounts of RNA as starting material and it is also very expensive. To study pathogen virulence gene expression in

vivo with microarrays it is often necessary to pool material from several hosts. Using real-time PCR, we have instead been able to compare the expression of selected *H. pylori* genes between infected individuals.

H. pylori virulence genes expression in the mouse gastric mucosa and in in vitro cultures (Paper III-IV)

Encouraged by our findings in vomitus and human gastric biopsies, we hypothesized that our approach to virulence gene expression analysis would be suitable for studying the expression of suggested vaccine antigens with the goal of identifying target antigens that are up-regulated in vivo. Not surprisingly, *cagA*, *flaA*, *hpaA*, *ureA* and *vacA* have all been proposed as vaccine target antigens (54, 100, 122, 154, 161). Since vaccine development requires extensive studies in animal models before going into human trials, we wanted to compare the virulence gene expression in the human biopsies to that in different stages in the mouse model, in in vitro cultured bacteria and finally in vomitus, as a suggested source of infection. For this purpose, we followed the expression of the previously studied genes over 14 weeks of infection in the mouse model and in early and late exponential phase and stationary phase in in vitro cultures. Again we found a pattern of relative virulence gene expression with *cagA*, *flaA* and *ureA* expressed at higher levels than *hpaA* and *vacA* both in the mouse gastric mucosa and in in vitro cultures. Surprisingly, *hpaA* was expressed at lower levels than *vacA* in the mouse gastric mucosa (Paper IV: Figure 3), which is in contrast to our findings in the human antral biopsies where it was expressed at nearly the same level as *cagA*, *flaA* and *ureA*. It is also unexpected in light of the previous study of the same *H. pylori* strain SS1 infection model in C57Bl/6 mice showing that *hpaA* is essential for colonization. This however shows that the number of gene transcripts per genome may not be strictly correlated to the importance of the protein and that analyses on the protein level are very important. We also found clear evidence of a general up-regulation of the analyzed genes during the late phase of exponential bacterial growth (Paper IV: Figure 3 and 4), i.e. between 1 and 2 weeks in the mouse model and at 24 h in in vitro cultures. When the colonization of the murine gastric mucosa reaches its plateau stage after approximately 2 weeks, all five genes are temporarily down-regulated. This down-regulation may be triggered in response to the onset of immune responses in the mice, although a similar down-regulation was found in the stationary phase of in vitro cultures, except for *flaA* which continues to be highly expressed even in the stationary phase. However, in the mouse mucosa the genes are up-regulated once more at 8 weeks, followed by down-regulation 14 weeks, even though the number of *H. pylori* recovered from the stomachs is

constant between 2 and 14 weeks (Paper IV: Figure 2). The down-regulation at 14 weeks may coincide with the onset of inflammation which occur in this mouse model after approximately 3-4 months (Sukanya Raghavan, personal communication). In conclusion, it seems likely that the studied virulence genes are up- or down-regulated to adapt to the host immune response and also depending on the rate of growth. Furthermore, since all five genes essentially show the same expression kinetics, it seems reasonable to assume that they are directly or indirectly controlled by the same factors or at the very least are simultaneously activated or repressed in response to internal and external signals. Furthermore, our analysis of *H. pylori* genes in the mouse model, in human gastric biopsies, vomitus and stool and in in vitro cultured bacteria show that the method is highly useful for studying bacterial gene expression in a wide range of sample types.

General discussion

Although *H. pylori* has been studied extensively over the last two decades, the transmission pathways of this pathogen remains elusive. *H. pylori* is a human specific pathogen, and its only natural niche is the human stomach and stomach-like epithelium in the duodenum. Considering that exposure to virtually any other conditions than those optimal for growth of this fastidious bacterium results in conversion into probably irreversibly non-culturable coccoids it is remarkable that this pathogen has been so successful in colonizing the human population. Transmission pathways have been analyzed in a number of studies which have indicated both direct person-to-person transmission and dissemination through various environmental sources. In line with several enteric pathogens a waterborne route has been proposed and tested in numerous studies which have provided evidence of a link to low sanitation and absence of clean water (76, 120). In this thesis we propose that the reported correlation between lack of clean water and *H. pylori* prevalence may be spurious or coincidental since *H. pylori* itself may not be present in infectious doses in drinking water. Instead we propose that a large fraction of new *H. pylori* infections in areas with high prevalence of *H. pylori* may be transmitted indirectly by other pathogens that cause gastrointestinal diseases with vomiting and vomitus-associated shedding of high levels of *H. pylori*.

Initially the overarching aim of this thesis was to study possible transmission pathways of *H. pylori* in Bangladesh. The rationale behind the project was the many reports of *H. pylori* traces in drinking and other water sources, but also the fact that none of these reports had been able to show evidence of actual transmission. We hypothesized that Dhaka, Bangladesh, with its high prevalence of H pylori infections, would be an ideal site to perform studies to link the presence of certain bacterial clones in both human and water samples as well as in newly acquired infections in children to prove the proposed hypothesis of waterborne *H. pylori*. To our surprise we were not able to amplify *H. pylori* DNA in any water samples including heavily contaminated environmental and waste water, despite using state-of-the-art technology and rigorous collection procedures and controls against false positive or negative results (Paper II). Studies on the same water samples for presence of ETEC on the other hand showed that ETEC was readily detected in as many as 67 % of the analyzed samples, simultaneously indicating that the methodology is adequate and that there is a risk of waterborne ETEC transmission (Paper I). We were thus forced to conclude that the risk of

waterborne *H. pylori* transmission in this area was lower than anticipated and unpublished work in another high prevalence area in Mozambique corroborated our conclusion.

However, during the same period, collaborative studies between our laboratory and the ICDDR, B showed that new infections of *H. pylori* in Bangladesh occur predominantly during the biannual peaks of acute infectious diarrhea that are caused by *V. cholerae* and ETEC (128). We had also shown that ETEC genome numbers were higher in drinking water during one of these peaks than in the intermediate, low endemic season. Therefore, we wanted to investigate if there was an increased risk of *H. pylori* transmission during the actual episodes of diarrhea. Previous research has also suggested that *H. pylori* may be spread during bouts of vomiting (14, 87, 89, 91, 126), a nearly ubiquitous symptom of ETEC and *V. cholerae* diarrheal diseases with frequencies of 81 % and 92 %, respectively (128). During the epidemic peak of diarrheal diseases in Dhaka in the fall of 2007, we found large numbers of *H. pylori* genomes in vomitus and approximately 1000-fold lower numbers in corresponding diarrheal stool (Paper III). Even though it was not possible to isolate the bacteria by culture, we could also show that the *H. pylori* in vomitus expressed several virulence genes, whereas *H. pylori* in stool lacked detectable gene transcription (Paper III). Furthermore, the data showed that the expression of these genes in vomitus was lower than in gastric biopsies from a comparable group of *H. pylori* infected individuals. Thus, the bacteria in vomitus may be infectious, but the lower number of transcripts per bacterial genome indicates that the bacteria are stressed or that only a fraction of the bacteria in vomitus are infectious. However, the infectious dose of *H. pylori* may be as low as 10^4 bacteria (55, 156) and the median *H. pylori* genome number in the vomitus samples was 4.35×10^5 ml^{-1}, indicating that many of the investigated vomitus specimens in our study may have contained an infectious dose of *H. pylori* in a fraction of a ml. Hence, the data strongly suggests that *H. pylori* may be spread during epidemics of acute infectious diarrhea and vomiting. Whether the putative transmission occurs directly from person to person or through some external source such as food or water remains to determined, but our previous research suggests that contamination of the water distribution system is unlikely. It seems more reasonable that transmission during these outbreaks occurs within households, especially in light of the previous studies suggesting transmission in families or close-living communities in both developing and developed countries. If this is the case, the actual transmission may occur through contamination of for instance hands or food, or transient contamination of water inside the household. Another possibility is that the infection is carried in aerosols formed during vomiting. With a worldwide annual diarrheal disease burden of between 2 and 4 billion cases, vomitus may constitute a substantial risk of

contracting *H. pylori*, especially in close-living communities, even if the overall vomitus frequency may be lower than that reported for ETEC and *V. cholerae*. Obviously, this could also at least partly explain the diminishing prevalence of *H. pylori* in developed countries, where diarrheal diseases are much rarer than in developing countries, often as a consequence to improved water sanitation. However, our study was small, comprising only 28 individuals, and it was carried out in a single location. More and larger studies in several geographic areas are required to find out if vomitus poses a true risk.

Increased knowledge of the mechanisms that are used by *H. pylori* to establish its colonization in the gastrointestinal tract of the host is important background information for vaccine development. In paper III and IV in this thesis the expression of five of the most important virulence genes of *H. pylori* were quantified in biopsies from infected individuals, in an experimental infection model as well as in a putative source of infection, i.e. vomitus from infected individuals. We used a novel approach of gene transcript quantification where we determined the absolute number of transcripts present per bacterial genome. This is a promising method that allows direct comparison of transcript levels in bacteria in different environments and may be used to determine various environmental influences on gene expression in an absolute quantitative manner. These studies showed that all five genes are expressed more or less universally in all these samples, with the exception of some vomitus specimens. It may further be noted that the genes were strongly up-regulated in the initial phase of colonization in mice, during which the bacteria essentially grow exponentially, indicating that the genes may be important for colonization. However, similar findings during the exponential phase of in vitro cultures suggest that the genes may always be up-regulated during bacterial replication. Regardless of what causes the up-regulation, our findings indicate that all of these genes may be interesting targets for prophylactic vaccine, since they are transcribed at high levels during the initial colonization of the mucosa. Environmental stress, such as stationary phase in vitro or after the onset of the murine immune response, seems to down-regulate the expression of these genes. Presumably, stress is also the cause of the lower absolute levels of virulence gene expression in the vomitus samples. Although *H. pylori* grown in vitro until late exponential phase are always used in experimental infection, additional research on the correlation of gene expression and infectivity is needed.

An important observation in our studies of *H. pylori* gene expression is that the relative expression of the investigated virulence genes is strikingly similar in human vomitus samples as compared to in gastric biopsies and in the established experimental *H. pylori* infection in

mice. Detailed studies of *H. pylori* and *H. pylori* gene expression in readily available vomitus samples from individuals in high endemic areas may thus give important information on the expression of key virulence factors in different populations and geographic areas. The similarities in relative gene expression further implies that the mouse model is suitable for studies of *H. pylori* virulence in vivo and of expression of putative virulence factors that could be targets for a vaccine.

In conclusion, we found that waterborne dissemination of *H. pylori* is less likely in Bangladesh and probably also in other high endemic areas as well while there may be a link between new infections and gastrointestinal infections that cause vomiting. We propose this hypothesis since large amounts of putatively viable *H. pylori* with highly similar transcript profiles of some of the most important virulence genes necessary for colonization of the stomach are present in vomitus. Hence this thesis has provided new evidence for the transmission and infection pathways of *H. pylori* which is one of the most common human bacterial pathogens worldwide. We further conclude that both the mouse model of *H. pylori* infection and vomitus from infected individuals may be used to study *H. pylori* virulence and gene expression.

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