PHENOTYPIC AND MOLECULAR ANALYSIS OF VIRULENCE GENES OF *Helicobacter pylori* ISOLATES FROM BANGLADESH

A DISSERTATION SUBMITTED TO THE DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY, UNIVERSITY OF DHAKA IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (SCIENCE)

SUBMITTED BY:

REGISTRATION NO. 104

YEAR 2001-2005

DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY

UNIVERSITY OF DHAKA

DHAKA-1000, BANGLADESH

CERTIFICATE

This is to certify that the thesis entitled "Phenotypic and molecular analysis of virulence genes of ***Helicobacter pylori*** isolates from Bangladesh" was carried out by Shamsun Nahar, Roll No. 104; Session: 2000-2001 for the fulfillment of the degree of Doctor of Philosophy (Science), from the Department of Biochemistry and Molecular Biology, University of Dhaka, Bangladesh.

This work was carried out under our supervision and the style and contents of the thesis have been approved and recommended for the award of Ph.D. degree.

Dr. Motiur Rahman
Head and Associate Scientist
RTI/STI Laboratory
Laboratory Sciences Division
ICDDR, B
Dhaka, Bangladesh

Professor Mamun Rashid Chowdhury
Department of Biochemistry and
Molecular Biology
University of Dhaka
Dhaka, Bangladesh

Abstract

ABSTRACT

Helicobacter pylori is a genetically diverse gram-negative bacterium that chronically infects more than half of all people worldwide and that is implicated in peptic ulcer disease and gastric cancer. Prevalence studies have indicated that *H. pylori* infection is extremely common in Bangladesh as in other developing countries. Most of our present understandings of *H. pylori* are based on studies of strains from the West and a few Asian countries. However, information on phenotypic characteristics, diagnostic methods, antimicrobial susceptibility pattern, virulence markers and genotypes of the Bangladeshi *H. pylori* strains were lacking. In this context we have characterized *H. pylori* strains to find out the scenario of *H. pylori* related consequences in these aspects in Bangladesh.

Diagnosis of *H. pylori* by non invasive test and to study the sensitivity of stool antigen test (HpSA) for the diagnosis of *H. pylori* infection was evaluated in 320 children aged 18-60 months with symptoms of gastritis was evaluated and compared with gold standard urea breath test (UBT). Of the 320 children, 196 (61%) were tested positive for *H. pylori* for *H. pylori* by UBT. Among these 191 subjects 153 was positive for *H. pylori* by stool antigen test (sensitivity 78%, specificity 99%). Of 124 children with negative UBT results, 123 were *H. pylori* negative in stool test (specificity 99%). The positive and negative predictive values of HpSA tests were 99% and 76% respectively. The concordance of HpSA test assessed by spectrophotometry and visual method was 100%, which makes the test even cheaper and easier. We conclude that stool HpSA test is reliable, rapid and easy way to exclude *H. pylori* infection in children in Bangladesh. The discrepancy between UBT and stool test with respect to *H. pylori* positivity requires further investigation.

Antibody response against *CagA* and *VacA* protein in children with *H. pylori* infection was evaluated. A total of 182 children aged 18-60 months were screened for *H. pylori* infection using UBT, and the serum samples were analyzed for antibody against *cagA* and *vacA* status by Western blot. The overall prevalence of *H. pylori* infection by 13C-urea breath test was 80%. The seroprevalence of *CagA, VacA* and both *CagA* and *VacA* were 82%, 82% and 81% respectively. Among children with a positive UBT,

95% were seropositive for both *CagA* and *VacA* indicating that the products of these genes are frequently co-expressed in *H. pylori* infection in this community. The sensitivity, specificity, positive and negative predictive value of the Western blot test for *H. pylori* infections, compared to UBT, were 94%, 68%, 92% and 76% respectively. The high seroprevalence of CagA and VacA positive virulent *H. pylori* strains in a symptomatic pediatric population indicate that such strains are common in this population and may cause characteristic *H. pylori* infection in Bangladesh.

The phenotypic and genotypic characteristics of *H. pylori* isolates from Bangladesh and its correlation with disease outcome was studied. A total of 278 consecutive patients between 15 and 78 years of age with symptoms of PUD and NUD were enrolled and gastric biopsy was cultured for *H. pylori*. Among the enrolled patients, 72.7% (202 patients) were male and 27.3% (76 patients) were female, 162 had PU and 116 had NUD and 62.6% (174 of 278) were culture positive for *H. pylori*. Among the culture positive patients, 121 (69.5%) were male and 53 (30.4%) were female and 112 (64.3%) had PU and 62 (35.6%) had NUD. There was no significance difference in smoking habit, alcohol consumption, family history and food habit in relation to disease presence in this population.

Antimicrobial susceptibility of 120 *H. pylori* isolates for metronidazole, tetracycline, clarithromycin, and amoxicillin was determined by agar dilution and E-test method. Among the isolates 77.5%, 15%, 10%, and 6.6% of the isolates, were resistant to metronidazole, tetracycline, clarithromycin, and amoxicillin respectively. The mechanism for metronidazole resistance was further studied in metronidazole susceptible isolates. Only rdxA inactivation and both rdxA and frxA inactivation were responsible for metronidazole resistance in 66% (8 of 12) and 33% (4 of 12) of the metronidazole susceptible isolates, respectively.

The virulence genes in *H. pylori* isolates from Bangladesh was studied and compared between isolated from PUD and NUD subjects. We compared several virulence markers (cagA, vacA, and iceA) and neutral markers (IS605, IS606, and IS608) in *H. pylori* strains isolated from 65 adult patients with peptic ulcer (PU) and 50 patients with nonulcer dyspepsia (NUD). cagA is present in 75% of the strains from patients with PU compared to 55% in patients with NUD, and 80% of the isolates from

patients with PU carried potentially toxigenic vacAs1 alleles of the vacuolating cytotoxin gene (*vacA*) compared to 60% in isolates from patients with NUD. However, no significant difference in any other virulence marker was observed in isolates from both groups. Phylogenetic analysis of the vacA middle region and the 5' end of the *cagA* gene indicates that Bangladeshi isolates are more closely related to *H. pylori* isolates from India and are different from isolates from East Asia.

The data of the present study will provide us with a basis for further characterization of *Helicobacter pylor*i isolates in Bangladesh, for better diagnosis and treatment purpose.

Table of Contents

List of Figures

List of Tables

LIST OF ABBREVIATIONS

CagA	Cytotoxin associated geneA
DU	Duodenal ulcer
DNA	Deoxynucleic acid
DATP	Deoxyadenosine 5'-triphosphate
dCTP	Deoxycytidine5'-triphosphate
dGTP	Deoxyguanosine 5'-triphosphate
dNTP	Deoxynucleotide triphosphate
dTTP	Deoxythymidine5"-triphosphate
GIT	Gastrointestinal tract
ELISA	Enzyme linked immunosorbent assay
et al	et aliii(and others)
Kb	Kilobase
KDa	Kilodalton
HSP	Heat shock protein
MALT	Mucosa associated lymphoid tissue
M	Molar
Mgcl2	Magnesium chloride
Mg	milligram
MM	Milli molar
nm	nanomole
OD	optical density
PCR	Polymerase chain reaction
PBS	Phosphate buffer saline
RUT	Rapid urease test
NUD	Non ulcer dyspepsia
TBE	Tris borate EDTA
UBT	Urea breath test
UGI	Upper gastrointestinal
VacA	vacuolating cytotoxin geneA

Chapter I
Introduction

Introduction

Helicobacter pylori is a Gram-negative, slow growing, microaerophilic spiral shaped bacterium that colonizes the gastric mucosa of 50% of the human population worldwide. It is a major cause of gastritis and peptic ulcer disease. Patients infected with *H. pylori* are 5 to 6 times more susceptible to develop gastric Adenocarcinoma and T- cell lymphoma. Humans are the principal reservoir of *H. pylori* infection. The prevalence of *H. pylori* infection varies widely by geographic area, age, race and ethnicity and in most of the cases the infection is asymptomatic. The prevalence of *H. pylori* infection appear to be higher in developing countries i.e. Asia (70 to 80%) and Africa (70 to 90%) than in developed countries i.e., North America (30 to 40%), Europe (30 to 70%) and U.S., 35–40% (122). The prevalence of infection in a symptomatic cases increases with age. In U. S. the infection rate is 20% by the age of 18 to 29 years while 47 % of over 60 years (59). In developing countries 40%-75% children are commonly infected by the age of 19 months (168).

Infection by *H. pylori* usually starts early in life, and persists for the rest of life unless eradicated by specific antibiotic treatment. *H. pylori* survive in the stomach as it produces the enzyme urease. Urease enables *H. pylori* to break down urea into ammonia and bicarbonate, the latter of which is then broken down into water and carbon dioxide. Ammonia alkalinizes the microenvironment that surrounds the bacteria as a protective device. *H. pylori* cannot survive in a highly acidic environment. However, *H. pylori* needs some acid in the environment to both grow and replicate, because these two processes are inhibited when the pH is more than 7.0. Because of their shape and the way they move, the bacteria can penetrate the stomach's protective mucous lining. Here, they can produce substances that weaken the stomach's protective mucus and make the stomach cells more susceptible to the damaging effects of acid and pepsin. The bacteria can also attach to stomach cells further weakening the defensive mechanisms of stomach and producing local inflammation. For reasons not completely understood, *H. pylori* can also stimulate the stomach to produce more acid.

Excess acid of the stomach and other irritating factors can cause inflammation of the upper end of the duodenum, the duodenal bulb. In some people, over long periods of

time, this inflammation results in production of stomach-like cells called duodenal gastric metaplasia. *H. pylori* then infect these cells causing further tissue damage and inflammation, which may result in an ulcer. *H. pylori* is usually present in at least 60-70% of gastric ulcer patients but it might be difficult to find in gastric ulcer patients because of concomitant atrophy and intestinal metaplasia (239).

Since the first discovery of *H. pylori* in 1982 by Warren and Marshal, it has been the topic of extensive research. A number of studies have used questionnaire components to investigate factors possibly related to the etiology of *H. pylori* infection. The exact mode of transmission of *H. pylori* is yet to be known but epidemiological data suggest person-to-person transmission, by either a fecal-oral, oral-oral, iatrogenic (from contaminated endoscopes machine) (172) transmission. Adequate nutritional status, especially frequent consumption of fruits and vegetables and of vitamin C, appears to protect *H. pylori* infection whereas, food prepared under less than ideal conditions or exposed to contaminated soil or water may increase the risk of infection.

H. pylori posses a number of virulence factors that enables *H. pylori* to survive inside the host as well as to produce disease. The most common virulence factors are flagella, *urease, vacA, cagA, babA, iceA, oipA* etc. Flagella help the bacteria to move in the mucus environment. Urease neutralizes the acid in the stomach. The cytotoxin-associated gene (*cagA*) gene is considered as a marker for the presence of cag pathogenicity island (cag-PAI) in *H. pylori* genome, which is a 40 kb locus in the chromosome containing 27 genes (184). *CagA* gene was initially found in association with the vacuolating cytotoxin (vac) gene. *CagA* gene is present in the majority of *H. pylori* isolates and its presence is associated with an increased risk of peptic ulcer disease and gastric cancer (247). The exact reason behind this is yet not known but is assumed that strain carrying *cagA* are more interactive with the host and produce more IL2 and IL8 than *cagA* negative strain (29). *VacA* is present in all *H. pylori* strain that induces massive vacuolar degeneration of various epithelial cell lines. *ice A*, a homologue of a gene for restriction endonucleases, induced by contact with gastric epithelium and OipA is a proinflammatory protein that contributes to IL8 induction independent to cag PAI.

In 1997 Tomb et al described the whole genome sequence of *H. pylori* 26695 containing 1,667,867 base pairs and 1,590 predicted protein coding sequences.

Sequence analysis indicates that *H. pylori* has well-developed systems for motility, scavenging iron, and for DNA restriction and modification. Many putative adhesins, lipoproteins and other outer membrane proteins were identified, underscoring the potential complexity of host-pathogen interaction. Based on the large number of sequence-related genes encoding outer membrane proteins and the presence of homopolymeric tracts and dinucleotide repeats in coding sequences, *H. pylori*, like several other mucosal pathogens, probably uses recombination and slipped-strand mispairing within repeats as mechanisms for antigenic variation and adaptive evolution. Consistent with its restricted niche, *H. pylori* have a few regulatory networks and a limited metabolic repertoire and biosynthetic capacity (234). An intriguing aspect of *H. pylori* is an extraordinary diversity among strains that is seen in DNA fingerprinting and in tests of gene content and chromosomal gene order together with this are indications of differences at certain loci between strains from different parts of the world or human ethnic groups.

Antimicrobial resistance is a growing problem for *H. pylori* infection. Resistance to metronidazole and other 5-nitroimidazoles has emerged worldwide and now constitutes a major problem in therapy (2). However, the prevalence of antimicrobial resistance varies with geographical regions (55). So continuous surveillance of antibiotic resistance to *H. pylori* needs to be monitored to determine regional trends and guide empirical treatment that ultimately helps to eradicate *H. pylori* infection.

In Bangladesh information on the prevalence, diagnosis, antimicrobial susceptibility, genotypes, of *H. pylori* as well as their clonality and insights into the population genetics structure are lacking. So, therefore we have tried to find out the scenario of *H. pylori* related consequences in these aspects in Bangladesh.

Chapter II
Literature Review

2.1 *Helicobacter pylori* -the organism

H. Pylori are a spiral shaped, gram-negative, non-spore forming microaerophilic rod usually with 4–7 flagella (Fig. 1). The flagella aid in the colonization of the bacterium to the gastric mucosa. The bacteria is 3–5 μm long and 0.5 μm in diameter, colonies of *H. pylori* from primary culture appear small, circular (1mm) convex, translucent and pale gray. *Helicobacter* species seem to be only bacteria that can survive in the harsh conditions in the stomach as it produces the enzyme urease. The organism is catalase positive. Prolonged survival is important clinically because it is the chronic infection that causes serious disease in tissue. The organism is curved whereas in culture they often are more rods like and bizarre U-shaped and circular cells are present. The wall of *H. pylori* is smooth and contains unusual fatty acids. Researchers believe that *H. pylori* are responsible for the majority of peptic ulcers. The bacteria (along with acid secretion) damage stomach and duodenal tissue, causing inflammation and ulcers.

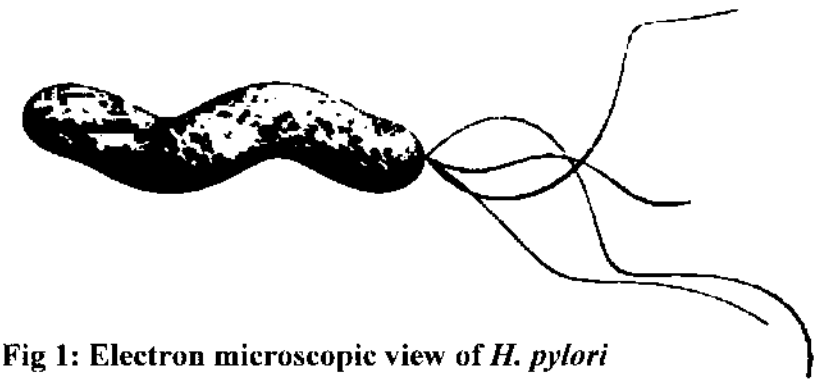


Fig 1: Electron microscopic view of *H. pylori*

H. pylori are a fastidious organism. The organism can be cultured from stomach biopsies or gastric juice. *H. pylori* require an enriched environment for growth. Many agar bases will sustain the organism if supplemented with blood, antibiotic supplementation (Dent) in the medium minimally increase culture sensitivity. Plates should be incubated at 37°C under microaerophilic conditions (10% CO_2, 85% N_2, 5% O_2). *H. pylori* grow optimally at 35 to 37°C; the optimum pH for growth is 6.8-7.5. However this organism can grow in wide pH range (5.5-8.5) (192). *H. pylori* grows slowly and plates may require 5-10 days incubation to develop recognizable colonies.

2.2 History

Although the association between *Helicobacter pylori*, gastritis and ulceration was discovered by Robin Warren in 1979 and the organism cultured by Barry Marshall in 1982, the historical origins of this manifold relationship date back to the halcyon epoch of microbe hunting in the late 19th and early 20th centuries (131). It is now widely apparent that *Helicobacter* is ubiquitous, pathological and, a century after its initial discovery, still remains a paradox of as yet incompletely determined biological consequence (Table 1).

Table 1: Historical overview of *H. pylori* (131)

1875:	Bottcher and Letulle discovered bacteria in the floor and margins of gastric ulcers; first hypothesis that bacteria caused ulcer disease.
1889:	Jaworski found "Spiral" bacteria in gastric aspirations.
1893:	Spirochetes were noted by Bizzozero in the gastric mucosa of dogs. These organisms were named *Helicobacter bizzozeronii* in 1996.
1896:	Mice successfully infected with *H. bizzozeronii* by Salomon.
1906:	Krienitz noted spirochetes in the gastric aspiration of a stomach cancer patient.
1921:	Edkins (the discoverer of gastrin in 1905) investigated the physiology of *Helicobacter felis* in the cat.
1938:	The association between spirochetes and gastric inflammation was noted in the *Macacus* monkey and in man by Doenges.
1940:	Freedberg and Barron "confirmed" that this organism had no etiologic role in gastric disease in man.
1940:	The hypothesis that an "acidophilic bacteria" caused ulcer disease was first postulated by Gorham.
1979:	*Campylobacter pylori* was identified as a putative causative agent of human gastritis by Warren
1983:	*C. pylori* was isolated and cultured by Marshall
1985:	Ingestion of *C. pylori* was demonstrated to cause gastritis in human volunteers.
1994:	NIH concludes that there is a strong association between *H. pylori* and ulcer disease and WHO classifies *H. pylori* as a group I carcinogen
1997:	Complete genome sequence of *H. pylori* released

2.3 Discovery and rediscovery

Over the past 20 years, research in mutually overlapping disciplines has demonstrated that *H. pylori* are only one member of a whole family of bacteria, which infect the entire gastrointestinal tract of both humans and animals. To date, at least 7 different types of *Helicobacter* have been isolated from the stomach alone and more are being identified in other organs of the gut. In 1982, Warren and Marshall provided the first insight into a new pathogenic factor in peptic ulcer disease. They isolated a urease-producing organism, later identified as *H. pylori*, nestling in the narrow interface between the gastric epithelial cell surface and the overlying mucus gel. In their early studies, the presence of this organism was shown to be highly correlated with antral gastritis as well as with gastric and duodenal ulcers, and eradication of this organism effectively eliminated ulcer recurrences. Furthermore, an epidemiological relationship between *H. pylori* infection and gastric malignancies was reported. In 1930 pathologists linked these organisms with gastric inflammation. In 1989 *Campylobacter pylori* was reclassified in a new genus *H. pylori*. Since 1983 considerable interest has focused on *H. pylori*, which were observed on the surface of gastric antral epithelium in patients with acute chronic gastritis, moreover infection has been linked to dyspepsia, peptic ulcer disease, hypertrophic gastropathy, gastric cancer and gastric lymphoma.

2.4 Prevalence and incidence

H. pylori infection is a common infection and is prevalent in both developing and developed countries. In contrast with industrialized nations, *H. pylori* infections occur earlier in life and with a higher frequency in the developing world. Also, while the prevalence of the infection has dropped significantly in many parts of North America, Western Europe and Asia (especially Korea), no such decline has been noted in the developing world (236). However, as in industrialized nations, gender does not appear to be related to infection with *H. pylori*. In many developing countries, the prevalence of infection with *H. pylori* exceeds 50% by 5 years of age, and by adulthood, infection rates exceeding 90% are not unusual (16, 66, 90). In a study of 569 Bangladeshi children between 2 and 10 years of age, the prevalence of *H. pylori* was 42% by 2 years of age and 67% by 10 years of age (211). Similar findings were reported from

studies performed on children from many parts of the developing world including Peru, Gambia and China (135, 174, 231). In a study of 200 Egyptian mothers tested from a project in Alexandria, Egypt, 90% were infected with *H. pylori*, and 15% of their infants were infected by 9 months of age, with the prevalence increasing to 25% by the time the children reached 18 months of age (18). A subsequent serosurvey using children participating in a 3-year study found that *H. pylori* infection increased with age and reached 30% by 3 years of age (180). Incidence data for infection with *H. pylori* are not as abundant as information on prevalence. However, few studies on the incidence of infection have been suggest that rates of infection in developing world are much higher than the 1–2% yearly incidence reported in industrialized settings. Additionally, as expected from the prevalence data, the incidence of infection with *H. pylori* is highest within the first few years of life. Among 186 Bolivian children serially tested over a 2-year period of time, seroincidence averaged 18% per year with a peak of 22% per year occurring in children between 2 and 3 years of age (89, 108).The finding is further supported by 1-year seroincidence of 24% among 112 Ethiopian children between 2 and 4 years of age (147). In a study conducted in 187 Egyptian children between 6 and 36 months of age participating in a longitudinal study of diarrhea, suggested that the incidence of *H. pylori* infection is 15% by the age 6-month (180). There are no studies providing data on the incidence rate of *H. pylori* infection among adults native to a developing country because they are almost universally infected with the bacteria. However, a unique group, adults from an area with a low prevalence of *H. pylori* who resided for an extended period of time in a high-prevalence country, provides an opportunity to study the risk in adults. While US troops sent to the Arabian Gulf during the Gulf War had a 1-year seroconversion of 14%, however, another study of 312 North American missionaries who were long-term residents (mean 7.4 years) of sub-Saharan Africa had an annual incidence rate of infection of only 1.9% (21, 115). The reason for the dramatic difference between the rates of infection between the two groups is not clear but may reflect different living standards including overcrowding, diet and water supply. Another ongoing question regarding infection with *H. pylori* is whether some infections are transient. In a serological study of Egyptian children between 6 and 36 months of age, 42% of the children had sero-reversion between the first and second blood test, suggesting a spontaneous clearance of the infection (180). Similar findings were reported from a cohort of Peruvian children diagnosed with *H. pylori* using the urea breath test (135).

The prevalence of infection was 71% in children 6 months of age but dropped to 48% by 18 months of age. Supporting these observations are several studies from developed countries, which have indicated that *H. pylori* infection may indeed be a reversible process. Thus while *H. pylori* infections in adults may be chronic, it appears that children may have repeated cycles of acquiring and losing the infection until the infection eventually becomes chronic, at least in developing country settings.

2.5. Source of infection

Despite many attempts at discovery, the exact source of *H.pylori* infection has yet to be determined. Contaminated food or water has been cited as important source for *H. pylori* infection. Laboratory experiments have found that *H. pylori* may survive up to a week in water (74). However; only one team has been able to culture the organism from an environmental water source (151). Using a multistep method, investigators cultured *H. pylori* from wastewater at a plant on the Mexican's border. While this report is unique till today, it certainly supports the theory that water may be a source of infection, especially in the developing world, where water treatment is often sub-optimal. Further support comes from epidemiological findings from the developing world. In a study of 407 children from Peru, an external water source was associated with a significantly increased risk of childhood infection with *H. pylori* (136). Compared to children using well water, children living in households with a municipal water supply had a 12-fold higher risk of being infected. The unexpected finding was thought to be due to breaks in the municipal pipes allowing for surface contamination of the water. A report from Bolivia also indicated that contaminated water is a common source of infection with *H. pylori* (89). Earlier studies have shown that children living in families using special water containers that prevented hands or objects from coming in direct contact with the family drinking water were significantly less likely to be infected with *H. pylori* than children whose family did not use the special water container. Such findings would support the thought that hands contaminated with *H. pylori*, rather than a contaminated water delivery system, transfer the bacteria to the drinking water. Unfortunately the study was not designed specifically to evaluate routes of transmission of *H. pylori*, thus no attempts were made to isolate the bacterium from the water sources. In a study of Chilean children,

consumption of uncooked vegetables was found to be associated with *H. pylori* infection, and it was postulated that this association resulted from ingestion of vegetables contaminated with irrigation water (109). A study from Bangladesh, however, was unable to show any relation between water source and risk of infection with *H. pylori*, suggesting that while water may be a source of infection, other sources likely exist (44).

2.6. Mode of transmission

Epidemiological studies strongly suggest person-to-person transmission, by either a fecal-oral or oral-oral route, as the major means of *H. pylori* transmission (142, 172). Reports from the developing world have further supported this hypothesis. In both China and Bangladesh, an increased number of individuals sleeping in a room were the factor most highly related to infection with the organism, suggesting a direct transmission through overcrowding (33, 44). Specific behaviors within the developing world may also contribute to the transmission of *H. pylori*. In Bangladesh, Hindu mothers, in contrast to Muslim mothers, regularly coat their nipples with saliva before breast-feeding (44) and it has been found that the prevalence of *H. pylori* is higher among the Hindu babies than among Muslim babies in rural Bangladesh. This could support the hypothesis of oral-oral transmission. Pre-mastication of food has been found in both Bangladesh and Ethiopia to be associated with an increased prevalence of *H. pylori* in babies, again supporting the possibility of oral-oral transmission (44, 148). Evidence supporting a fecal-oral spread of the organism has recently been reported in an interesting study from Central America. Using PCR-based technology, investigators were able to amplify *H. pylori* DNA from oral secretions of study subjects (63). Scrapings from underneath the fingernail were also taken from the study subjects, and *H. pylori* DNA was detected in 58% of the samples. Furthermore, *H. pylori* were more likely to be found in the mouth of a study subject if the bacteria were found under his/her nails. The housefly, a common resident of homes in the developing world, has been touted as a potential mode of transmission of *H. pylori*. Previous studies have demonstrated that houseflies are associated with transmission of enteric infections including *Shigella*, raising the possibility that flies may also transmit *H. pylori*. Initial controlled laboratory experiments demonstrated *H. pylori*

could be cultured from the body of flies up to 12 hours after being in contact with pure colonies of the organism (95). Additionally, *H. pylori* could be cultured from the gut and excrement of the fly for up to 30 hours after the fly was exposed to the bacteria. In a subsequent study, using flies caught at Egyptian field sites, *H. pylori* was detected from the gut of 33% of the pools of houseflies tested (96). While neither study proves the ability of the fly to transmit *H. pylori* nor how common this transmission method would be, these intriguing preliminary findings may warrant further study.

2.7 Risk factors

Poverty-related factors have repeatedly been found to be associated with an increased risk of acquiring *H. pylori*. These factors include overcrowding, poor hygiene unclean water source, sharing beds during childhood and lack of maternal education (58, 148, 161, 179). Other factors are potentially associated with an increased risk of acquiring *H. pylori*, including family members with *H. pylori* infection, pre-mastication of food by the mother, not breast-feeding, low education level in parents, and possibly, contaminated food, and race/ethnicity. The overwhelming poverty of many parts of the developing world has prevented the elimination of the risk factors for *H. pylori* and helps to explain the high prevalence of *H. pylori* infection.

While socioeconomic factors are strongly associated with the risk of acquiring *H. pylori*, genetic and behavioral variables also influence the risk of becoming infected with *H. pylori*. Studies in Malaysia showed that throughout the country, under similar economic and living conditions, people of Malay ancestry were significantly less likely to be infected by *H. pylori* than Chinese and Indian populations in the same region of Malaysia (91). A second study of asymptomatic children of different ethnic backgrounds but all born and raised in Belgium showed that the Caucasian children were significantly less likely to be infected than the non-Caucasian children (143). However, other studies within industrialized settings have not been able to reproduce these findings, suggesting that while there may be true racial or ethnic predispositions to infection with *H. pylori*, the findings could also be explained by unrecognized environmental or behavioral differences (158, 159). Further work in this area is necessary.

There are indications that behavioral practices can influence the risk of transmission of *H. pylori*. Pre-mastication, still common in parts of the developing world, has been linked with an increased risk of transmission of *H. pylori*. After an initial report from Bangladesh, a subsequent study in Ethiopia also made similar observation that maternal premastication of food increased the risk of transmission of *H. pylori* (44). Of the eight Ethiopian mothers who chewed the food before feeding it to their children, seven of these offspring were infected with *H. pylori* compared to 37 of 69 children being infected if their mothers did not pre-chew their food (149). While pre-mastication could be a surrogate marker for other factors, it lends support to the idea that direct oral-oral spread of *H. pylori* is possible. While breast-feeding has been shown to decrease the risk of infections in infants, especially from diarrheal disease, it is unclear whether the practice affects the risk of acquiring *H. pylori*. One difficulty in discerning the relationship between breast-feeding and infection is the fact that breast-feeding is almost ubiquitous in the developing world till one year of age. Studies in the United States showed breast-feeding have a protective role against acquisition of *H. pylori* (160). However, other studies were unable to demonstrate a protective effect of breast-feeding and one study even found that it increases the risk of childhood infection (61, 133, 215). Further evaluation demonstrated that the increased risk was associated with the mother not washing her nipples and hands prior to breast-feeding. Direct transmission of *H. pylori* to the infant, rather than lack of protection from breast milk, likely explains this finding and is consistent with the hypothesis that direct transmission of *H. pylori* occurs by mothers coating their nipples with saliva prior to breast-feeding.

2.8 Virulence factors

Helicobacter pylori posses a number of virulence factor that help the organism to survive inside the stomach and to produce disease (49). Well-known virulence factor are urease, LPS, flagella, CagPAI, *VacA*, P-type ATPase, heat shock protein, OipA (Table 2).

Table 2. Reported virulence factors of *H. pylori*

Factor	Function	Distribution
Urease	Buffers stomach acid	All strains
Flagella	Motility	All strains
NAP	Neutrophil activation	All strains
BabA	Adhesin for LewB	Prevalent on type I strains
LPS	Low toxicity	All strains
Lewis^{x,y} antigens	Molecular mimicry	Some strains
IceA	Homolog of Nla III restriction endonuclease	Some strains
VacA	Cytotoxicity (two alleles)	All strains
cag PAI	31 genes coding for type IV secretion system	Type I strains
CagA	Immunodominant antigen (part of *cag* PAI)	Type I strains
PicB	Equivalent to CagE	Type I

2.8.1 Urease

H. pylori urease enzyme is unique among bacterial ureases. The urease enzyme consists of six copies each of the structural subunits, *UreA* and *UreB*, and two-nickel ions reside in each of the six active sites. The *ure* gene cluster encodes the two structural subunits (ureA and ureB) and five accessory proteins (*ureI, ureE, ureF, ureG, and ureH)*. The accessory proteins are required for nickel ion insertion into the apoenzyme (177, 220). Urease is one of the most abundantly produced proteins by *H. pylori*. This enzyme helps protect the bacteria from gastric acid by the production of ammonia, which increases the pH of the microenvironment around the organism. Direct injury to gastric epithelial cells occurs in vitro from ammonia (223). Ammonia exposure may impede gastric cell cycle progression, causing a delay in cells from progressing through the S-phase in the cell cycle (164). Studies of Eaton and

Krakowka have shown that using isogenic mutant *H. pylori* strains without urease activity fails to colonize gnotobiotic pigs, even when acid secretion is suppressed (67, 68, 69). These studies showed the critical role of urease in the pathogenesis of the bacteria and suggested that the ammonia produced may be a nitrogen source for the synthesis of proteins necessary for bacterial adhesion.

2.8.2 Flagella

H. pylori possess 4-6 sheathed flagella. Flagellum, which is essential for swimming in to the gastric mucosa, consists of flaA and flaB. At least forty proteins in the *H. pylori* genome appear to be involved in the regulation, secretion and assembly of the flagellar architecture. As has been reported for the flaA and flaB genes and identified sigma 28 and sigma 54- like promoter elements upstream of many flagellar genes, underscoring the complexity of the transcriptional regulation of the flagellar regulon (176).

2.8.3 Cag Pathogenecity Island (Cag PAI)

CagA, a gene that codes for an immunodominant antigen, is present only in *H.pylori* strains that are associated with severe forms of gastroduodenal disease (type I strains). The genetic locus that contains Cag Pathogenicity Island (cagPAI) is a 40-kb locus containing 27 genes inserted in the chromosomal glutamate racemase gene of *H. pylori*. Its G+C content (35%)differs from the rest of the genome (39%), suggesting that it was acquired from another organism by horizontal transfer (38). This pathogenicity island is flanked by direct repeats of 31 bp (Fig 2). In some strains, cag PAI is split into a right segment (*cagI)* and a left segment (*cagII*) by a novel insertion sequence (*IS605*). In a minority of *H. pylori* strains, cagI and cagII are separated by an intervening chromosomal sequence. Nucleotide sequencing of the 23,508 base pairs that form the cagI region and the extreme 3' end of the cagII region reveals the presence of 19 ORFs that code for proteins predicted to be mostly membrane associated with one gene (cadge), which is similar to the toxin-secretion gene of *Bordetella pertussis*, *ptlC*, and the transport systems required for placid transfer, including the virB4 gene of *Agro bacterium tumefaciens.* Transposon inactivation of several of the cagI genes abolishes induction of IL-8 expression in gastric epithelial

cell lines. Thus, it is believed that cag region may encode a novel *H. pylori* secretion system for the export of virulence determinants.

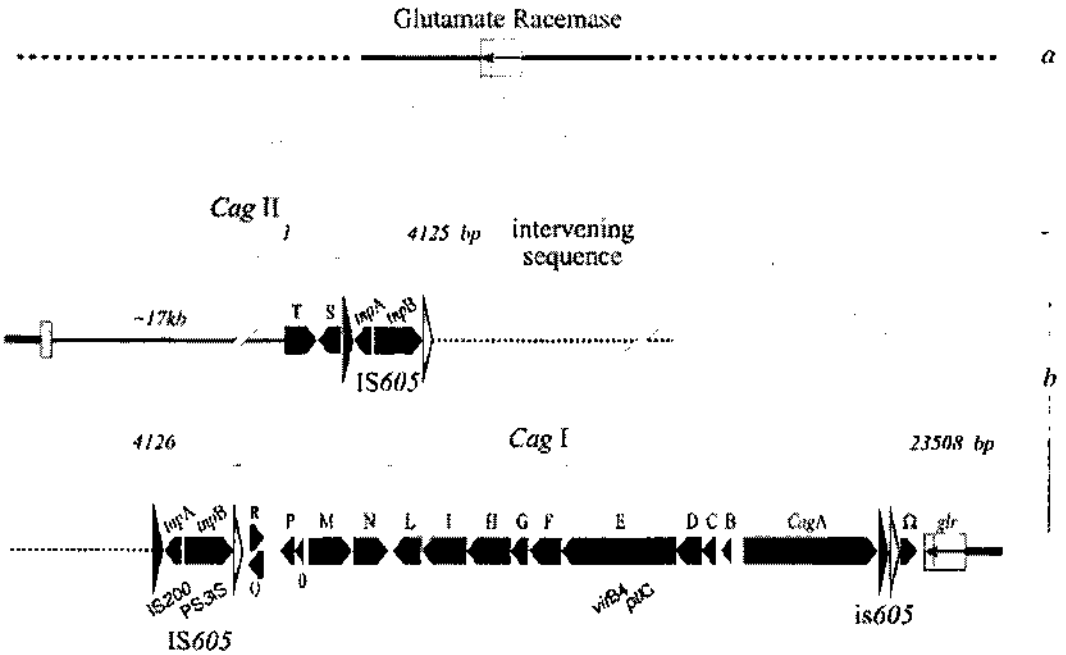


Fig 2. Schematic representation of the cag region as deduced from analysis of strain CCUG 17874. The cag integration site within the glutamate racemase gene (glr) is shaded (a). cag structure: the putative ORFs are represented by arrows (b). Flanking the intervening sequence (dots), the IS605 have a left and right end indicated with large solid and open arrowheads. (38)

Strains with partially deleted cag PAIs may be cagA positive or negative, and even though they represent an intermediate form, they would be scored as type I or type II strains based on this feature. An important consequence of the interaction between *H .pylori* and host epithelial cells is the elevated production and secretion of cytokines, such as interleukin-8 (IL-8) (252).

An active toxin is produced only by type I strains; however, the linkage between CagA and VacA expression is not yet clear since the *cagA* and *vacA* genes are located more than 300 kb apart on the chromosome of the *H. pylori* NCTC 11638 (34). Moreover, it has been shown that inactivation of the *cagA* gene has no consequence on expression of *VacA* or on the ability to induce IL-8 (34, 52, 238). *CagA* is a marker for the increased virulence observed in type I strains.

2.8.4 VacA

All *H. pylori* strains contain a copy of vacuolating cytotoxin gene (*vacA*), which encode a protoxin with a mass of about 140 kDa that is flower shaped (49) and causes vacuolar degeneration of gastric epithelial cell by interfering with intracellular membrane fusion. More recently the toxin has been shown to reduce transepithelial resistance by loosing tight junctions (49).

Sequence analysis of *vacA* alleles from different *H. pylori* strains have shown that there are more pronounced differences between alleles in some region of the gene than in others i.e. a region near the 5' end of the gene is relatively well conserved between alleles. Within this region, recombination has destroyed virtually all-detectable Phylogenetic clonal structure and there appears to be linkage equilibrium between most loci. The portion of *vacA* that exhibits maximum diversity is an approx 800bp "mid region" which encodes part of the 58kda domain of *vacA*. In contrast to the region near the 5' end of *vacA*, sequences from the mid region can be phylogenetically divided into two families of alleles termed m1 and m2. Strains with evidence of recombination in the mid region between m1 and m2 alleles appear rare, with m1/m2 recombinant alleles described only in a few Chinese strains of *H. pylori*. Strains with evidence of recombination in the mid region among m1/m2 alleles are more frequent but recombination has not been sufficient to abolish phylogenetic structure within the m1 and m2 types. Aside from the *vacA* mid region various other smaller region differ markedly between *vacA* alleles. In particular the signal peptide region encoding part of the amino terminal signal peptide, exhibits striking diversity of strains. Two main allelic families of signal regions are recognized as s1 and s2 and s1 can be further subdivided into s1a, s1b, and s1c. The combinations are s1a/m1, s1b/m1 s1c/m1 and s1a/m2 etc. This ratio is similar for comparisons among m1 mid regions and m2 mid regions, but higher for comparison between m1 and m2 regions. Strains possess *vacA* s1m1 alleles are more toxigenic than *vacA* s1m2 allele (49, 176).

2.8.5 Association between cagA and vacuolating activity

The close but not absolute association between the presence of the cytotoxin associated gene *cagA* and vacuolating activity has been enigmatic. In particular, insertion mutagenesis of *cagA* or deletion of other genes in the cag pathogenicity island (PAI) does not alter vacA expression or activity. The discovery of different *vacA* allelic types has gone some way to explain the link that there is a close genetic association between the presence of cagA (and other genes in the cag PaI) and the presence of type s1 alleles. However, given the high level of intraspecies recombination in *H .pylori* and the separation of *vacA* and the cagPAI on the *H. pylori* chromosome, the reason for this genetic association is unclear. It seems likely that there is some as yet unidentified selective advantage to strains that is conferred by either possessing or lacking both type s1 *vacA* and the cagPAI. *H. pylori* produce a toxin that has damaging effects on epithelial cells and *H. pylori* colonization is a strong risk factor for the development of peptic ulceration (Fig 3).

2.8.6 The iceA gene

Although there is not much evidence for iceA as a virulence factor of *H. pylori*, the expression of iceA is induced by contact of *H. pylori* with human epithelial cells. The gene has a structure similar to a restriction endonuclease gene from *Neisseria lactamica* and exists as two distinct types, *iceA1* and *iceA2*. The frequency of *iceA1* was found to be about 50 % in duodenal patients and very rare in non-ulcer patients. The function of iceA remains unclear. (77). *IceA1* is highly associated with duodenal ulcer in comparison with *iceA2*.

2.8.7 The babA gene

The babA gene encodes the outer membrane protein that mediates binding of *H. pylori* to the blood group antigen Lewis B on the human host cell surface. It exists in two distinct genotypes, *babA1* and *babA2*, *babA1* comprises an almost complete ORF but lacks the necessary initiation codon and is therefore not translated. In contrast babA2 contains a 10-bp insert at its 5' end that includes an inframe ATG start codon.

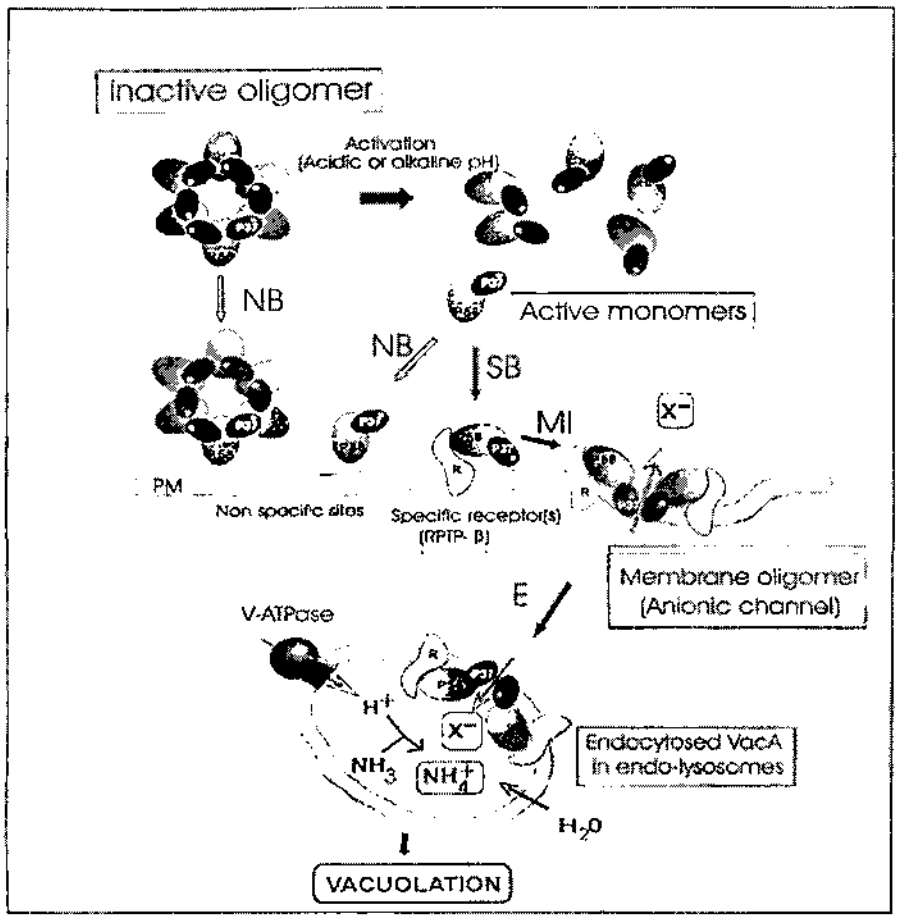


Fig 3. A possible model for the interaction of *Vac A* with the epithelial cell.

The inactive oligomer made up of p37 and p58 fragments, can be disrupted by acidic or alkaline treatment to release active monomer *Vac A*. **Binding**: Both the oligomeric and monomeric toxin are thought to undergo non-specific binding (NS) to the plasma membrane (PM) which probably does not result in endocytosis. Only activated toxin is thought to undergo specific binding (SB) to VacA receptor(R), which may allow toxin internalization. **Membrane-interaction (MI)**: Hydrophobic VacA monomers can insert into the plasma membrane where they associate to from a pore. The membrane-associated toxin is an anion (X) selective Channel, which allows exchange in ion flux at the level of the plasma membrane. **Endocytosis (E)**: Membrane-associated Vac A is internalized, presumably by receptor-mediated endocytosis, transported and accumulated in endolysosomes. **Vacuolation:** Anion conduction by endocytosed Vac A channel stimulates electrogenic proton pumping activity of the V-ATPase. In the presence of ammonia (or other weak bases) this may lead to ammoniun ion accumulation in the endolysosomal compartment. The increased concentration of osmotically active ammonium ions is believed to lead to water influx, swelling of endolysomoes and vacuole formation.

This permits expression of the babA2 ORF, which correlates with bacterial adhesion. A part from the 10bp insert, the sequences of *babA1* and *babA2* are highly similar. A strong association between *cagA*, *vacAs1* and *babA2* was found in patients suffering from peptic ulcer (85, 116).

2.8.8 The oipA gene

The *H. pylori* outer inflammatory protein, OipA, is an important virulence factor associated with enhanced interleukin- 8 secretion and increased inflammation in vitro as well as the clinically important presentation of peptic ulcer (254, 255, 257). Expression of OipA is regulated by the slipped-strand repair mechanism based on the number of CT dinucleotide repeats in the 5' region of the *oipA* gene (switch on-functional and switch off-nonfunctional) (255), such that gene switch status may affect bacterial characteristics such as virulence. Isolates that contain the *cag*PAI (CagA is a marker) typically also have OipA with functional status "on" (10, 38, 256). Currently, prediction of the presence of a functional OipA protein requires PCR-based sequencing of the signal region of the gene.

2.8.9 Neutrophil Activating Protein (NAP)

Neutrophil Activating Protein (NAP) is an oligomeric protein made of 10-12 copies of a 17kd of polypeptide with homology to iron binding proteins. This protein has a capacity to activate neutrophils and may be involved in the recruitment of this cell to the gastric mucosa and hence nay contribute to the inflammatory response (49).

2.8.10 LPS and Lewis antigens

The lipopolysaccharide of some *H. pylori* strains contains structures identical to the fucosylated Lewis x and Lewis y blood group antigen expressed on the gastric mucosa. This antigenic mimicry may result in immune tolerance against antigen of the pathogen or in induction of autoantibodies that recognize gastric epithelial cells, frequently observed inpatient with chronic active gastritis (49).

2.9 The *H. pylori* Genome

In 1997, the whole genome of *H. pylori* strain of 26695 was sequences by Tomb et.al. He first reported that it has a circular genome of 1,667,867 base pairs and 1,590 predicted coding sequences (Table 3). Sequence analysis indicates that *H. pylori* has well-developed systems for motility, for scavenging iron, and for DNA restriction and modification. Many putative adhesins, lipoproteins and other outer membrane proteins were identified, underscoring the potential complexity of host-pathogen interaction. Based on the large number of sequence-related genes encoding outer membrane proteins and the presence of homopolymeric tracts and dinucleotide repeats in coding sequences, *H. pylori*, like several other mucosal pathogens, probably uses recombination and slipped-strand mispairing within repeats as mechanisms for antigenic variation and adaptive evolution. Consistent with its restricted niche, *H. pylori* have a few regulatory networks, and a limited metabolic repertoire and biosynthetic capacity. Its survival in acid conditions depends, in part, on its ability to establish a positive inside-membrane potential in low pH.

H.pylori is one of the most genetically diverse of the bacterial species, with any given isolate easily distinguished from most other isolates by simple PCR based DNA fingering or limited DNA sequencing (4, 150). This extra ordinary diversity is due variously to differences among strains in gene content and gene arrangement, to numerous point mutations within individual genes, and to rich history of combination between strains (1). Some of the diversity may be adaptive, affecting variously the chance of successful transmission in a physiologically diverse human population, the chance that an infection will persist and the chance that persistence will lead to the overt disease.

The publication of the complete genome sequence of two *H. pylori* strains (strains 26695 and J99) provided many insights into *H. pylori* biology (6, 234). Each of these genomes consist of a single circular chromosome about 1.7Mb in size, similar to that have *H. influenzae* and one- third to one fourth the size of *E. coli* or *vibrio cholerae.* This small genome size reflects a limited metabolic repertoire and biosynthetic capacity, consistent with the specialization of *H. pylori* for growth in unique gastric niche. Nearly 1600 protein coding sequence (genes) was identified in each genome.

About 100 strain but not the other. Nearly 500 lack obvious homology to other genes in the database (114). Perhaps some of these species- specific are critical for *H. pylori* colonization of the stomach.

The *H. pylori* genome seemed to code fewer global regulatory proteins than do *E. coli* type models. For example, no homologous of the OxyR, SoxR or SoxS oxidative stress regulators nor of the LexA –SOS DNA damage response regulator have been found (24). This might reflect exposure of *H. pylori* to a smaller number of environments than other organisms encounter. However, homologous of other known regulatory genes have been found such as nickel responsive transcriptional repressor gene (54, 234), ferric uptake (22) carbon sugar regulator gene (234), RNA polymerase (sigma 54 factor) (224) catalase gene (189), HSP homologous- GroEL, GroES, DnaK, DnaJ, GrpE, OmpR (113, 121, 229, 234) and homolog of luxS, whose product catalyses the synthesis of an autoinducer that may regulate quorum sensing (80, 120) . This array of potential regulatory genes is in accord with the consideration that it may encounter environmental changes, such as varying pH levels, progressive immunological responses and possibly nutrient fluctuations and appropriate gene regulation may be critical for successful adaptation and persistent colonization of human stomach. Many *H. pylori* genes showed stronger amino acid level homology to genes known from gram-positive organisms, Archeae or eukaryotes than to homologs of other gram-negative organization (234). This suggests considerable gene transfer from disparate phylogenetic groups into *H. pylori* lineages. Two distinct but related insertion sequence (IS) elements IS605 and IS 606 are present in each of the two sequenced genomes and in some one fourths of strains overall (107, 126, 234). Additional members of this group, IS607 (128) and ISH p608 (130) have been found recently in other *H. pylori* strains. Each of these IS elements is present in only a subset of *H. pylori* strains and such sporadic distributions constitute evidence of gene transfer during evolution. Each element contains two genes orfA, which may be of distinctly different phylogenetic origin, and orfB. Recent mutational tests with IS607 (128) and IS608 have shown that only orfA, are needed for transposition in *E. coli* and thus probably encode the transposase. In contrast, ofrB is a homolog of a *Salmonella* enterica gene that is needed for its invasion in Peyer's patches. This leads to the interesting possibility that the IS elements of *H. pylori* might contribute to its human host interactions.

Many families of duplicate and often divergent genes have been found in *H. pylori*. Of particular interest is a 35-member family encoding putative outer membrane proteins (OMP). Several members of the family encode adhesions (babA, hopZ), (116, 201) one contribution to the inflammatory response in cell culture and perhaps disease in vivo (OipA) (255) and several others seem to encode porins (hopA-E) (65). Different alleles of yet another duplicate OMP gene (omp5, omp29) seem to be selected in different human hosts. There is sufficient DNA homology among some members of this family to allow recombination, which would generate novel chimeras that might differ in receptor specificity, antigenicity or other feature important in host interaction. Nine of the OMP genes have polymeric tracts (polyCT, polyA, or polyT) near their 5' ends and could easily undergo frame shift mutations (gain or loss of one or two repeat units), thereby switching metastably between ON and OFF states (234). Such genes are called contingency' genes; it is believed that their expression may be selected for and against at various times during infection. Then ON/OFF metastability would help ensure that some members of any population could exploit new niches or survive certain adverse conditions.

The *H. pylori* genome is also extra ordinary in containing homologs of some two dozen DNA restriction and modification(r/m) gene complexes (11, 137, 234, 253). Some seem to be complete; others may be vestigial (no longer functional); and their exact complement varies among strains. Although their roles are not known, several models merit consideration. In one model, r/m systems may be molecular parasites (selfish DNAs), whose presence reflects evolutionary accident. A second view focuses on the restriction feature of r/m systems in which DNA breakage would interfere with the exchange of large DNA segments, so stimulate exchange of short DNA segments (DNA ends are excellent recombination substrates). A third view focuses on how methylation (modification) of transcription promoters decreases promoter activity, thereby affecting gene expression. Changes in expression of sets of co-adapted genes might then be achieved through gain, loss or changes in activity of particular methylase genes. A fourth possibility came with the discovery of *iceA1*, a gene whose transcription seems to be specially induced by *H. pylori* contact with gastric epithelial cells (202) and whose carriage seems to be virulence associated in Western strains. This gene, when functional, encodes a restriction endonuclease suggesting the possibility of *H. pylori* restriction endonuclease acting directly on host tissue.

Many *H. pylori* clinical isolates carry plasmids whose sizes range from 1.5 to >40kb.Three small plasmids have been completely sequenced, putative replication genes have been identified (102, 134) and *H. pylori-E. coli* shuttle vectors have been developed. Based on precedents from other soecies, it is expected or hoped that some of the genes in larger plasmids will affect *H. pylori* colonization, persistence or virulence.

2.9.1 Genetic manipulations

The molecular genetics of *H. pylori* has developed rapidly the last decade, and a number of genes that may affect colonization or the nature and severity of disease have been found. Of immense value in this regard have been the DNA sequencing of the genomes of the two representative strains from ethnic Europeans strains 26695 and J99(7). Very useful methods for genetic manipulation have been implemented for *H. pylori*, including efficient reverse genetics by DNA transformation and allelic replacement, reporterer genes, shuttle vector plasmids, and new genome micro array and proteome technologies (46, 76, 97, 103, 153). There are also excellent cell culture models (116, 124) and animal models for analysis of bacteria-host interactions, especially inbred mice of diverse genetic constitutions, specific transgenic mice and Mongolian gerbils. Also used have been domestic cats beagle dogs, hamsters, gnotobiotic piglets, rhesus monkeys and Japanese macaques (105, 132, 139, 144, 163, 249). Equally important in this context is our increasing understanding of human gastric physiology a growing awareness of the great diversity among *H. pylori* strains, and a corresponding diversity among humans in traits that may be important to individual strains.

2.10 Sequelae of *H. pylori* infections

H. pylori infection has been shown to be associated with chronic gastritis, peptic ulcer disease and gastric cancers. Associations with chronic gastritis and peptic ulcers are more commonly reported in the developed world, and gastric carcinoma is more commonly found in the developing world (35). Reasons for this difference is not clear and may be related to reporting.

Table 3: Genome features of *Helicobacter pylori* (234)

General	
Coding regions (91.0%)	
Stable RNA (0.7%)	
Non-coding repeats (2.3%)	
Intergenic sequence (6.0%)	
RNA	
Ribosomal RNA	Coordinates
23S-5S 445,	306–448,642bp
23S-5S	1,473,557–1,473,919 bp
16S	1,209,082–1,207,584 bp
16S	1,511,138–1,512,635bp
5S	448,041–448,618 bp
Transfer RNA	
36 species (7 clusters, 12 single genes)	
Structural RNA	
1 species (ssrD)	629,845–630,124 bp
DNA	
Insertion sequences	
IS605 13 copies (5 full-length, 8 partial)	
IS606 4 copies (2 full-length, 2 partial)	
Distinct G + C regions	Associated genes
region 1 (33% G + C) 452–479 kb	IS605, 5SRNA and repeat 7; *virB4*
region 2 (35% G + C) 539–579 kb	cag PAI (Fig. 4)
region 3 (33% G + C) 1,049–1,071 kb	IS605, 5SRNA and repeat 7
region 4 (43% G + C) 1,264–1,276 kb	b and b9 RNA polymerase, EF-G (fusA)
region 5 (33% G + C) 1,590–1,602 kb	two restriction/modification systems
Coding sequences	
1,590 coding sequences (average 945 bp)	
1,091 identified database match	
499 no database match	

An alternative theory is that disease presentation is related to the age at which an individual is infected with *H. pylori* (30). Infections early in childhood are postulated to induce a low-grade chronic inflammatory condition, which, over time, develops into pre-malignant changes and eventually gastric carcinoma in a subset of individuals. In contrast, when infection is acquired later in life, it is more likely to induce a brisk inflammatory response and ulcer disease. The "African enigma" indicates that *H. pylori*-associated pathology is more complicated than described

above (140). The so-called "enigma" is that in many parts of Africa the prevalence of infection with *H. pylori* is high and most infection is acquired early in life. However, in the same setting, gastric carcinoma is unexpectedly low. Since only 10–15% of patients infected with *H. pylori* are thought to become symptomatic and because of concern about high rates of re- infection, especially within the developing world, it is not currently recommended to treat a symptomatic *H. pylori*. Epidemiological studies suggested that *H. pylori* might produce significant sequelae in children even before symptoms of gastritis or peptic ulcer develop. Pathologic evidence suggests that *H. pylori* infections of children in less-developed settings can produce rapidly destructive gastric mucosal lesions, which could conceivably destroy the gastric acid barrier even during childhood (82, 236). Achlorhydria may persist for months after acute *H. pylori* infections in adults, and evidence suggests that the condition may last up to a year in children (20, 101). As the acid barrier of the stomach constitutes an important host defense against enteric pathogens, destruction of the barrier could render children more vulnerable to diarrheal diseases and adverse nutritional sequelae. The relationship between infection with *H. pylori* and enteric infections was demonstrated in a study from Bangladesh, in which persons infected with *H. pylori* were found to be more likely to develop severe cholera (45). Studies from India have recently reported an association between infection with *H. pylori* and the development of typhoid fever (25). Children from the Gambia with chronic diarrhea were found to be twice as likely to be infected with *H. pylori*, and studies in Peru indicates that children acutely infected with *H. pylori* were at an increased risk of developing diarrhea (198, 227). However, other investigators have been unable to show an association between infection with *H. pylori* and increased diarrhea rates in children (36, 117). The reason for the discrepancy between the results is not certain but is likely multi-factorial, including different study methodology, as well as host and environmental factors.

The long-term sequelae of repeated or chronic diarrhea in children in the developing world include malnutrition, growth failure and death. Studies in Gambia showed that children infected with *H. pylori*, in addition to being more likely to develop chronic diarrhea, were also at a significantly higher risk of malnutrition than a group of children not infected with *H. pylori* (227). Infection with *H. pylori* may even affect growth in well-nourished children from industrialized areas. Studies among Scottish school children showed that *H. pylori*-infected girls had mild growth failure when

compared to uninfected controls (199). The association between infection with *H. pylori* and diminished growth in children, however, remains unsettled. No association between infection with *H. pylori* and poor nutritional status was found in a group of Bangladeshi children, but this may be related to the high baseline rate of malnutrition in that population (44). Future research supporting the finding that *H. pylori* is associated with the development of diarrhea could have important public health ramifications for the developing world, including decisions about the use of vaccines to control *H. pylori* in these settings.

2.11 Re-infection with *H. pylori*

Since *H. pylori* infection rates are much higher in the developing world than in industrialized countries, it has been presumed that re-infection rates with the organism following successful eradication therapy would also be higher than the 1% rate documented in the developed world. Data from Bangladesh, Chile and Peru support this theory. In a study in Bangladeshi, 105 patients with duodenal ulcer disease were followed up to 2 years after successful eradication therapy (104). Using urea breath tests as the diagnostic tool, re-infection rates of 13% per year were documented, with the majority of re-infections occurring within the first year. Similarly, of 96 Chilean patients who successfully had their infections eradicated, 12 (13%) had endoscopes confirmation of the infection returning, and in nine of 12 cases, infection recurred within a year of completing therapy (214). However, neither of these studies was able to differentiate re-infection from recrudescence. One study that attempted to discriminate between these two possibilities was performed in China (173).

An important finding of this study was that only four of the 184 patients (1.1%) were re-infected over the 2 years of observation, and of them 75% (3/4) become re-infected within 6 months of completing therapy. Additionally, molecular analysis of *H. pylori* isolates from the three patients with apparent re-infection documented that one of the three cases was actually a recrudescence rather than a true re-infection. The reason for the differing results between the Chinese study and the other two reports is not clear but indicates further data needs to be collected to address the issue. Although the re-infection rate may indeed be higher in the developing world than in industrialized

settings, data still support the recommendation that incorporating *H. pylori* treatment into standard therapy for peptic ulcers is warranted. However, if further studies in the developing world consistently document the high rate of recurrence after eradication therapy, treatment of a symptomatic *H. pylori* infection within the developing world would probably not be warranted.

2.12 Role of *H. pylori* in peptic ulcer disease

The strong association between *H. pylori* infection and the later development of duodenal ulcers is based on several lines of evidence. First, patients with duodenal ulcers who do not take nonsteroid anti-inflammatory agents (NSAIDs) are likely to be infected with *H. pylori*. Earlier studies found that >90% of duodenal ulcers were caused by *H. pylori*, although more recent carefully controlled studies have found that, in the absence of NSAIDs, *H. pylori* is the cause of duodenal ulcers in 70% of cases. Second, patients with duodenal ulcers infected with *H. pylori* are unlikely to have recurrence of their disease if treated for *H. pylori*. Patients with duodenal ulcers have nearly a 70% chance of recurrence if they are treated only with acid suppressants, while patients treated with acid suppressants and *H. pylori* eradication have a <10% chance of recurrence. Third, the natural history of *H. pylori* infection of the stomach involves a mechanism that predisposes one to duodenal inflammation and ulceration. When patients develop *H. pylori* infection, inflammation occurs predominantly within the antrum; the body of the stomach is relatively spared. The resultant gastritis leads to increased stimulation of G-cells, which release gastrin, a hormone that stimulates enterochromaffin-like cells to release histamine. When histamine is released, it binds to histamine-type 2 receptors on parietal cells, which then release acid. Over time, this constant stimulation leads to an increased acid load delivered to the duodenum, which results in mucosal irritation. If the infection is not eradicated, or if the acid load is not suppressed, duodenal ulceration can ensue.

Much of the evidence used to support the role of *H. pylori* in the development of duodenal ulcers also applies to gastric ulcers. That is, *H. pylori* is known to produce gastritis; eradication of *H. pylori* results in resolution of gastritis and patients with *H. pylori* treated with antibiotics have significantly lower rates of gastric ulcer recurrence

as opposed to those individuals who did not receive antibiotic therapy. In the absence of NSAID use, *H. pylori* are associated with gastric ulcers 60% of the time.

H. pylori infection resulting in duodenal ulcers, can also result in gastric acid hypo secretion if the infection is chronic in nature. Infection with *H. pylori* in these patients is usually more extensive and produces inflammation within the entire body of the stomach, a condition called pan gastritis. This chronic inflammation leads to atrophy of the mucosa of the stomach body, the area containing the acid-secreting parietal cells. Gastrin secretion in response to a meal still occurs; however, the number of functional parietal cells remaining is quite low, thus leading to reduced gastric acid secretion. With time, G-cells may also be lost, and thus gastric acid secretion declines even further. Chronic atrophic gastritis is one step in the pathway of the development of gastric cancer.

2.13 *H. pylori* and gastric cancer

Gastric cancer is the fourth most common cause of death from cancer in the UK (after lung, colorectal and breast cancer) and accounts for about 10,000 deaths each year in the UK. Five-year survival after resection is not particularly good, being perhaps 5% for all patients and 20% for those having a potentially curable resection (225). Sue-Ling et al, 1993 showed that gastric cancer could be diagnosed at an earlier stage through more widespread use of endoscopes, and that treatment of early stage cancers results in excellent five-year survival rates (above 70% for stages I and II, compared with 30% for stage III cancers). A prospective study investigating dyspeptic patients over 40 years at their first visit to a general practitioner with early endoscopes covering 10 general practices in Birmingham (99) demonstrated a 16% prevalence of peptic ulcer and a 2% prevalence of gastric cancer. A high proportion of the gastric cancers found were operable and a significant proportion discovered sufficiently early to be curable. An association between *H. pylori* infection and an increased risk of gastric cancer was suggested first by histopathological examination of gastric biopsy specimens showing that *H. pylori* infection was more common in patients with gastric cancer than in those with no pathological lesions. A case-control study (79) which looked at serum antibodies from a relatively small number of index cancers showed

not only was there a higher rate of seropositivity in cancers than controls (69% vs. 47%), but that antibody concentrations in the cancer patients were considerably higher (90 μg/mL vs. 3.6 μg/mL). The Forman study estimated an odds ratio for risk of gastric cancer in those infected with *H. pylori* of about 2.8 (95% confidence intervals 1.04 to 7.97) (79). Carcinoma of the stomach is the second most common cancer worldwide, and adenocarcinoma of the stomach accounts for nearly 95% of all gastric cancers. In 1991 Parsonnet et al. (197) first described the association of *H. pylori* with the development of gastric cancer. This association is now well accepted, and *H. pylori* is presently classified by the World Health Organization as a type I carcinogen (IARC Working Group). The evidence used to support this association includes the following: *H. pylori* is commonly present (>80% of cases) in histological samples of tissue adjacent to gastric carcinoma; analysis of stored serum samples has shown that there are higher seropositive rates for *H. pylori* in patients who eventually develop gastric cancer than in those patients who never develop gastric cancer (odds ratio of 3.6) (212) and long-term *H. pylori* infection in an animal model has been shown to induce gastric adenocarcinoma. Furthermore, the incidence of gastric adenocarcinoma has declined markedly in the Western world over the last 50years after *H. pylori* treatment. This decline mirrors a decrease in the prevalence of *H. pylori* and atrophic gastritis. Gastric cancer is thought to evolve from *H. pylori* infection in the following manner: *H. pylori* infection produces an active chronic gastritis, leading to chronic atrophic gastritis (over decades), which leads to intestinal metaplasia followed by dysplasia, ultimately leading to gastric adenocarcinoma. Although the association between *H. pylori* infection and later development of gastric carcinoma is clear, it is important to emphasize that gastric adenocarcinoma is uncommon in the U.S. (only 25,000 cases per year) and that any one individual infected with *H. pylori* has only a 1:40,000 chance of developing gastric adenocarcinoma. In certain countries, such as Colombia and China, where *H. pylori* infects over half the population in early childhood, gastric cancer is the most prevalent form of cancer (185). According to the National Institutes of Health (NIH) about 24,000 people in the United States are diagnosed with stomach cancer each year. Of these, about 14,000 will die from the disease. Data gathered from population studies had previously found an association between *H. pylori* infection and later development of a number of diseases, including 1) gastric mucosa-associated lymphoid tissue (MALT), 2) MALT lymphoma, 3) gastric cancer, 4) pancreatic cancer, 5) ischemic heart disease, 6) ischemic

cerebrovascular disease, 7) atherosclerosis, 8) periodontal disease, and 9) skin diseases such as rosacea. (10) They usually occur later in life with a peak in the seventh decade, with men more commonly affected than women. The association between *H. pylori* and MALT lymphomas has been verified by many studies over the last decade (186). *H. pylori* are believed to act as an antigen in the initiation of an immune response. T-cells respond vigorously to the initial infection, and cytokine production (interleukin-2, interleukin-10 and -interferon) is increased. Over time, the character of the inflammatory response changes, as most MALT lymphomas are ultimately composed of B-cells. Histologically, they may be identified as low grade (less common) or high grade (more common) and appear quite similar to Peyer's patches. Initial treatment aimed at eradicating *H. pylori* infection resulted in eradication of the MALT lymphoma also in 76% of patients (191).

If data from smaller studies are combined and summarized, 75.4% of patients with MALT lymphoma have complete regression when treated for *H. pylori* (195). Nonulcer dyspepsia or gastritis nonulcer dyspepsia (NUD), also called functional dyspepsia by many, is a disorder of the upper gastrointestinal tract present for >3 months, for which a biochemical or structural disorder cannot be found. Common symptoms include upper abdominal pain, nausea, and bloating. NUD occurs more frequently in younger patients (<25 years of age) and is equally present in men and women. There is considerable overlap of symptoms with irritable bowel syndrome. NUD is a heterogeneous condition, and researchers have attempted to categorize the symptoms into four separate classes: ulcer-like, reflux-like, dysmotility-like, and finally, nonspecific dyspepsia. The role of *H. pylori* infection in the pathogenesis of NUD is not entirely clear. The rate of *H. pylori* infection in patients with NUD is quite high—up to 87% in some studies. A meta-analysis has shown that subjects with NUD are twice as likely to be infected with *H. pylori* as control subjects. Several studies have demonstrated that *H. pylori* infection often precedes the development of symptoms however, specific symptoms attributable to *H. pylori* in the NUD group have not been found. Several theories have been proposed to account for the relation between *H. pylori* infection and NUD. These theories include the effects of *H. pylori* on gastric acid secretion and gastric motility. Acid secretion has been found to be higher in patients with NUD who are infected with *H. pylori*, as compared with those who are *H. pylori* negative. One study reported that patients with NUD who were *H.*

pylori positive had delayed gastric emptying, which improved after eradication of *H. pylori*. However, most studies have not demonstrated any consistent relationship between gastric emptying and *H. pylori* infection. Theoretically, *H. pylori* infection, acting through a generalized inflammatory response, may make patients with NUD more sensitive to normal gut stimuli. Eradication of *H. pylori* in patients with NUD has provided mixed results. One meta-analysis found that 73% of patients with NUD who became *H. pylori* negative after treatment noted an improvement in symptoms, compared with only 45% of patients who remained *H. pylori* positive. A second meta-analysis, however, found that eradication of *H. pylori* did not improve symptoms of NUD (210). Treating *H. pylori* in patients with NUD has been shown to be cost-effective, and may reduce potentially unnecessary endoscopes. Dyspepsia, defined as pain or discomfort centered in the upper abdomen is a common clinical problem. Numerous hypotheses have been suggested as to the cause of symptoms in patients with nonulcer dyspepsia, including perturbations of gastro duodenal motility, hypersensitivity to physiologic stimuli including acid, and the effects of infection within the gastric mucosa by *H. pylori*. Some epidemiological studies have suggested that patients with NUD may have a slightly higher prevalence of *H. pylori* infection. However, association does not prove causation. Causation of NUD by *H. pylori* could best be documented by resolution of symptoms following eradication of the infection. Early intervention studies indicated that there was a beneficial effect on symptoms of NUD with *H. pylori* eradication, but most of these studies had serious methodological flows. In the last few years there have been a number of well-designed studies investigating the effect of *H. pylori* eradication on symptoms in patients with NUD. The results of these studies are inconsistent, but suggest that there is little, if any benefit from treatment (75). Gastro-oesophageal reflux disease (GERD) and *H. pylori* infection are common conditions that frequently coexist (188). Controversy continues regarding the role of *H. pylori* infection in GERD. The results of some studies suggest that eradication of *H. pylori* may increase the risk for developing GERD, and some experts have suggested that chronic *H. pylori* infection may be of benefit (241) reflux of gastric acid from the stomach into the esophagus is the major event in patients with GERD. Several lines of evidence show that *H. pylori* may actually protect against severe acid reflux in patients with GERD. It is well known that *H. pylori* infection of the stomach may produce chronic gastritis. In time, this results in a decrease in gastric acid production, as previously described. A persistently low level of gastric acid

production will minimize the amount of acid reflux. Epidemiological studies have shown that patients with GERD often have a lower incidence of *H. pylori* infection than patients without GERD, and that eradication of *H. pylori* may lead to the development of acid reflux disease in some patients. Although these data support the notion that *H. pylori* may actually protect or minimize the effects of acid reflux disease, most clinicians favor the eradication of *H. pylori* once found because of its potential to increase the risk of developing gastric carcinoma or MALT lymphoma. A variety of abnormalities contribute to the development of GERD including transient lower esophageal sphincter relaxation, low esophageal sphincter pressure, presence of a hiatal hernia, diminished esophageal clearance of refluxed gastric contents, and alterations in esophageal mucosal resistance. *H. pylori* infection clearly plays a role in the pathogenesis of peptic ulcer disease and mucosa associated lymphoma of the stomach and is a definite risk factor for distal gastric cancer. The role of *H. pylori* infection in GERD remains controversial and incompletely understood. Although *H. pylori* infection does not cause reflux disease, circumstantial evidence suggests that it may protect against the development of GERD and its complications in some patients. The most likely mechanism whereby *H. pylori* infection protects against GERD is by decreasing the potency of the gastric reflux ate in patients with corpus predominant gastritis. A variety of implications of *H. pylori* infection on GERD treatment have also arisen in recent years. These focus on the risk of gastric atrophy while on proton pump inhibitor therapy and the efficacy of proton pump inhibitors before and after eradication of *H. pylori*. This article puts into perspective our current understanding of the complex, incompletely understood relationship between *H. pylori* infection and GERD (73). The incidences of GERD and esophageal adenocarcinoma have increased in recent years as the incidence of peptic ulcer disease and distal gastric cancer has declined. Given the simultaneous decline in *H. pylori* infection, it is tempting to propose a relationship between *H. pylori* infection and these opposing time trends. Although *H. pylori* infection clearly does not cause GERD, it may protect certain susceptible individuals from developing GERD and its complications. The most likely mechanism in which *H. pylori* infection protects against GERD is by decreasing the potency of the gastric reflux in patients with corpus predominant gastritis. A variety of implications of *H. pylori* infection on GERD treatment have also arisen in recent years. These focus on the risk of gastric atrophy while on proton pump inhibitor therapy and the efficacy of proton pump inhibitors before and after eradication of *H.*

pylori (72). Barrett esophagus is a metaplastic condition that affects the lower esophagus and is a complication of GERD. Under normal circumstances, a complex barrier at the esophagogastric junction prevents the reflux of gastric contents into the esophagus. Dysfunction of the lower esophageal sphincter and the presence of a hiatal hernia lead to failure of this barrier. Esophageal mucosal damage results from the chronic exposure of the esophageal mucosa to gastro duodenal contents and the lack of an effective mucosal defense. Gastric adenocarcinoma is still the second most common cause of death from cancer, even though it is on the decline in developed countries. Although *H. pylori* gastritis appears to be a necessary antecedent to the development of gastric adenocarcinoma, it is not a sufficient factor in and of itself. Other required factors for the progression of this disease are poorly understood. Patients with antral predominant gastritis seem protected from the disease, while patients with pan gastritis are predisposed to both diffuse- and intestinal-type adenocarcinoma. Development of a vaccine against *H. pylori* might yield promising results in decreasing the incidence of gastric adenocarcinoma (206).Although a complete understanding of the organism and the nature of its association with disease is incomplete gastritis appears predispose to gastric ulcers because the damaged cells are not susceptible to acid and peptic digestion. *H. pylori* colonizes the human gastric mucosa and establishes a chronic infection that is tightly associated with atrophic gastritis, peptic ulcer, gastric carcinoma, and adenocarcinoma.

2.14 Effect of aspirin

Despite great proven and potential benefits, aspirin also has adverse side effects on all parts of the gastrointestinal tract including ulceration, bleeding, perforation, and steno sis. Because of widespread and growing use, there is a need to understand and, if possible, prevent the adverse effects of aspirin while maintaining its benefits. Non-steroidal anti-inflammatory drugs (NSAIDs) and *H. pylori* are well-recognized causes of gastro duodenal mucosal damage. This damage is mediated through the effects of both agents on acid secretion, neutrophil activity and function, and prostaglandin metabolism. Clinical trials on the interrelation- ship between *H. pylori*, NSAIDs and gastro duodenal mucosal injury have yielded conflicting results.

No consensus has been reached on what recommendations should be implemented with regard to *H. pylori* eradication in patients on long-term NSAID therapy. At

present, the presence of *H. pylori* is identified at endoscopes and eradication is carried out in symptomatic patients. A symptomatic patients remain a dilemma that requires further investigation (17).

2.15 Diagnosis

When choosing the appropriate test, it is important to consider the cost of the test, to determine if the patient has previously been treated for *H. pylori*, and to find out if the patient is on other medications that might influence test results i.e., antibiotics, bismuth and proton pump inhibitors (PPIs). Six methods are now routinely used to diagnose *H. pylori* infection (Table 4).

Serology: Most laboratories now use an enzyme-linked immunosorbent assay (ELISA) to check for IgG antibodies. This test is inexpensive, and sensitivity and specificity are estimated to be 80–90%.

This method cannot, however, differentiate between a current infection and previous exposure. After treatment and eradication, antibody levels remain positive for years, although titers may drop by 50% at 12 months.

Table 4: Diagnosis methods of *H. pylori* infection.

Type	Test	Specimen
Non -invasive	UBT	Breath
	Stool Antigen	Test Stool
	Immunoblot	Serum
Invasive	RUT	Biopsy
	Culture	Biopsy
	Histology	Biopsy

pH indicator tests: These tests are performed at the time of upper endoscopes. The test strips (e.g., CLO test, PyloriTek, Hpfast) check for the presence of urease.

Sensitivity and specificity are high (95–98%). Recent antibiotic, bismuth, or PPI use (within 2 week) may produce a false negative result. The urease tests themselves are inexpensive; however, they all require the additional expense of endoscopes.

Histology: At the time of endoscopes, biopsies are taken from the ant rum and usually from the fundus as well. The presence of a chronic active gastritis strongly suggests infection, while the absence of chronic active gastritis virtually excludes infection. Various stains can be used (hemotoxylin and eosin, Giemsa, Warthin-Starry) to identify *H. pylori*. Sensitivity and specificity are high (95% range). Biopsy specimens are moderately expensive, although the cost of the endoscopes makes this an expensive test.

Culture: This is reserved for those patients who have been treated for *H. pylori* infection in the past but have had a recurrence. Biopsy samples are submitted to the laboratory for culture and determination of antibiotic resistance. This is a cumbersome and expensive process.

Breath test: These are now available and can be performed in the office. Patients are given a small amount of radioactively labeled carbon (^{13}C or ^{14}C) coupled to urea. The urease breaks down the urea, producing radioactive bicarbonate. This is absorbed through the gastric mucosa and then broken down into $^{13}CO_2$ or $^{14}CO_2$, which can be measured as the patient breathes into a bag. The dose of radioactivity is low less than one-twentieth of a chest X-ray. The sensitivity and specificity are high (>95%), while the cost is moderate. This test is best used to determine *H. pylori* eradication after treatment. However, patients must be off all proton pump inhibitors 7–14 days in advance of the test and need to be off bismuth products and antibiotics for at least 4 week.

Stool antigen test: These are new and became commercially available in the last year. They appear to be accurate (80 - 94% specificity and sensitivity) and reasonably priced. Antibiotics, bismuth products, and PPIs may all decrease sensitivity (39, 162, 167, 190).

2.16 Antibiotic sensitivity

Antibiotics in most of the developing world have led to an increased level of resistance of bacteria to many of the commonly used antibiotics, and *H. pylori* have been no exception. Antibiotic resistance data for *H. pylori* is not available from much of the developing world, but that which is available shows disturbing trends (8, 12, 71, 84, 208). The greater than 50% rate of resistance of *H. pylori* isolates to metronidazole has rendered this antibiotic nearly useless for the treatment of the organism. Although not certain, the high resistance to metronidazole is thought to be related to the frequent use of metronidazole to treat parasitic infections such as giardiasis and amebiasis. A most dramatic example of metronidazole resistance has been found in one of our ongoing studies of *H. pylori* among children evaluated in the gastroenterology-clinic. Of the 46 children from whom an isolate of *H. pylori* was obtained, none of the isolates were sensitive to metronidazole, with a mean inhibitory concentration of greater than 256 μg/ml(8, 12, 71, 208). An interesting report from Japan found that over only a 3-year period, *H. pylori* resistance to clarithromycin and metronidazole doubled, and the increase in resistance paralleled the increased use of these antibiotics within the country (203). Thus, judicious use of antibiotics along with obtaining data on the antibiotic resistance patterns for *H. pylori* within a country, and potentially within regions of a country, is critical for selecting an appropriate treatment regimen.

A study on the efficacy of therapy among 138 Israeli patients infected with *H. pylori* clearly demonstrates the need for selection of antibiotic therapy based on resistance patterns (216). Resistance to metronidazole and clarithromycin was significantly higher in patients who failed antibiotic treatment than in those who achieved a cure. Eradication of *H. pylori* is much less likely if the patient fails the initial therapy, often due to antibiotic resistance with lack of alternative therapies, making it all the more critical to have an effective first-time therapy.

Industrialized settings routinely report successful treatment in up to 90% of cases with as short as a 7-day course of therapy (243). However, it appears that treatment success is not as likely within the developing world, and a longer length of therapy is

required. In a study in Brazil using a standard treatment regimen of amoxicillin, clarithromycin and omeprazole, overall cure rates of only 64% were achieved, and the rates dropped to 50% if less than 10 d of therapy were used (123). Multiple factors are likely involved with the poorer treatment outcomes within the developing world but, at a minimum, the data suggests that treatment regimen from industrialized countries as well as the reported successes in that setting may not necessarily be translatable to the developing world.

2.17 Treatment

Twenty years ago, stomach ulcers were treated by drinking cream, so loaded with fat and cholesterol that it could cause heart attacks. Today, almost everyone with belching and burning in the stomach should be treated with antibiotics. In 1983 they laughed at Dr. Barry Marshall when he reported that stomach ulcers were caused by infection with *H. pylori* and could be cured with antibiotics. Fellow physicians were so mean to him that he responded by swallowing a vial of *Helicobacter* and almost died. Triple therapy, combining a proton pumps inhibitor with clarithromycin (C) and either amoxicillin (A) or a nitro-imidazole (I) is the standard in *H. pylori* eradication therapy. Recently, triple therapies based on ranitidine bismuth citrate (RBC) have emerged as an alternative. This review examines the current literature for studies directly comparing proton pump inhibitor- with RBC-based triple therapies. Eradication rates in an intention-to-treat analysis ranged from 51 to 98%. Table 5 summarizes the FDA approved treatment regimen for *H. pylori*.

No large difference in cure rates between the different regimens was demonstrated, although the RBC-I-C combination was somewhat superior. No definite conclusions could be made about the impact of metronidazole or clarithromycin resistance since only three studies performed a formal resistance analysis. No serious side effects were reported, and drop out rates were equal for the two regimens. Both RBC- and proton pump inhibitor-based triple therapies are highly effective. If one prefers an imidazole/clarithromycin combination the evidence presented here suggests that RBC should be used instead of a proton pump inhibitor. Larger studies comparing both forms of triple therapy, using proper resistance analysis, are needed before final conclusions can be reached regarding efficacy in the setting of bacterial resistance (246).

Table 5: FDA Approved treatment regimen [http://www.cdc.gov/ulcer/md.htm]

Omeprazole (40 mg daily) plus clarithromycin (500 mg three times daily) For 2 weeks, then omeprazole (20 mg daily) for 2 weeks
-OR-
Omeprazole (20 mg twice daily) plus clarithromycin (500 mg twice daily) Plus amoxicillin (1 g twice daily) for 10 days
-OR-
Lansoprazole (30 mg twice daily) plus clarithromycin (500 mg twice daily) Plus amoxicillin (1 g twice daily) for 10 days
-OR-
Lansoprazole (30 mg twice daily) plus amoxicillin (1 g twice daily) plus Clarithromycin (500 mg three times daily) for 10 days
-OR-
Lansoprazole (30 mg three times daily) plus amoxicillin (1 g three times daily) for 2 weeks*
-OR-
Esomeprazole (40 mg daily) plus clarithromycin (500 mg twice daily) plus Amoxicillin (1 g twice daily) for 10 days
-OR-
Ranitidine bismuth citrate (400 mg twice daily) plus clarithromycin (500 mg three times daily) for 2 weeks, then ranitidine bismuth citrate (400 mg twice daily) for 2 weeks
-OR-
Ranitidine bismuth citrate (400 mg twice daily) plus clarithromycin (500 mg twice daily) for 2 weeks, then ranitidine bismuth citrate (400 mg twice daily) for 2 weeks
-OR-
Bismuth subsalicylate (525 mg four times daily) plus metronidazole (250 mg four times daily) plus tetracycline (500 mg four times daily) for 2 weeks plus H2-receptor antagonist therapy as directed for 4 weeks

2.18 Vaccines

Perhaps the most exciting work in the quest to eradicate *H. pylori* as a significant human pathogen is in the area of vaccine development. The fact that the organism is prevalent worldwide, is responsible for significant morbidity and mortality, and is difficult and expensive to eradicate makes it a prime target for vaccine therapy. Pioneering work in the early 1990s provided evidence that vaccination against *H. pylori* infection was possible, based on murine models. It was later learned that the key mechanism of protective immunity against the organism occurred via stimulation of T-helper type2 phenotype cells, which are induced by the production of interleukins 4 and interleukins 10 and not by antibody production.

Several issues remain in regard to a safe and effective vaccine against *H. pylori* infection. In the first place, a safe mucosal adjuvant or vector to stimulate an immune response must be identified. Different agents, including cholera toxin and an *Escherichia coli* heat labile toxin, have been used in conjunction with specific *H. pylori* antigens (e.g., urease) with varying success (and varying toxicities) (62, 171). Attenuated live vaccines, including strains of *Salmonella*, used in combination with *H. pylori* antigens have shown promise (47). Secondly, the optimal route of administration needs to be defined; studies in mice show promise with nasal and rectal routes, which would avoid the possible post immunization gastritis likely with an oral route(228). In addition, different regimens need to be developed to ensure complete sterilization of the gastric mucosa; the latter step has not generally been attempted in murine models. Clinical trials are underway with the goal of producing an inexpensive, safe, and effective vaccine seeming to be within reach.

2.19 Genetic diversity and population structure of *H. pylori*

One of the most intriguing aspects of *H. pylori* is its genetic diversity, which is at a level as yet seen among other bacterial pathogens. A plethora of molecular typing techniques is available, may be of which have been applied successfully to distinguish between isolates. Alignment of representative orthologue sequences of various housekeeping genes such as *ureC, flaA, flaB* and *cysS* from any given pair of *H. pylori* strains indicate 3-5% diversity, with most nucleotide substitution occurring at

synonymous sites (83, 121, 226). But for several other *H. pylori* including *vacA* (14, 193, 245), *hspA* encoding GroES homolog, (121), and *cag A* (242), much of the genetic diversity among strains represent non- synonymous nucleotide substitutions which may reflect selection for different structures, functions or antigenic properties. Homoplasy tests have indicated extensive recombination among *H. pylori* strains (1), most probably in the settings of sporadic and transient mixed infection. Given mutational diversity, this recombination creates additional diversity among strains. Anecdotal evidence suggests that some 5-10% or more infections are mixed which provide opportunities for genetic exchange between strains. In terms of possible functional significance of this genetic diversity, it has been proposed- (i) that people differ in traits important to individual *H. pylori* strains (ii) that in consequence individual *H. pylori* strains tend to adapt and evolve during the years of chronic infection, becoming progressively better adapted to their individual hosts and (iii) that also in consequence, many strains will be less perfectly suited to the next host (s) happening to ingest them. Hence most new infections should kick off new cycles of selection for adaptive changes, further diverge from their ancestral strains. Especially given mixed or recurrent infection, much of *H. pylori*'s adaptation could involve lateral gene transfer between co-resident strains with complementary genotypes and then selection of any recombinants that grow better than either parent.

In addition to the general diversity among *H. pylori* strains in a given community are indications of differences at certain loci between strains from different parts of the world or human ethnic groups (128, 178). In particular DNA sequence motifs predominating in the virulence associated genes, *vacA*, *cagA* and *iceA*, in strains from Europe and US are found to differ from those predominating in East Asia. For example, strains from East Asian and non-Asian countries can be distinguished by differences in several different parts of *cagA*.

Biogeographically diversity is also observed for two fragments of *vacA* termed the signal(s) sequences and the mid region (m) (244). While each of *vacA s1*a allele type and *vacA*s1b type are common in European and US population, more than three-fourths of East Asian strains carry the *vacA* s1c. Even among the East Asian strains, nearly all-Japanese *H. pylori* carried *vacA*m1 type alleles while most Chinese strains harbored the m2 alleles. The regional differences in *cagA* and *vacA* alleles might reflect

natural selection, random genetic drift or both during *H. pylori* evolution. Phylogenetic clustering was also, observed in sequences of some housekeeping genes including *cysS*, *recA*, *picA*, *picB*, *ppA* and *atpA*. Also, in these cases, isolates from East Asia and West from distinct clusters.

The distribution of informative markers in *H. pylori* strains in different human population have helped to determine the possible evolutionary origin of this bacterium as a human pathogen. There is a disagreement over how long *H.pylori* has infected humans; in one-view *H. pylori* have been with our primate ancestors and us for millions of years (28). Alternatively, it has been proposed that it is a late invader, perhaps having jumped from animals to human hosts only in the last ten thousand years (128). Strains from Spaniards, Peruvians and Guatemalan Ladinos (mixed European-Amerindian ancestry) possessed similar RFLP patterns for a locus from Cag Pathogenecity Island and differed markedly from East Asian strains. Amerindians migrated from Siberia and were geographically separated from the Europeans until several hundred years ago. If their Siberian ancestors carried *H. pylori* that is related to East Asian isolates, then some alleles related to East Asian alleles should have been detected. Possibly the first Amerindians might have been *H. pylori*-free because they were hunter- gatherers, not agriculturists, and indeed had crossed from Asia to the Americas well before animal based agriculture began. They probably acquired these bacteria after the arrival of Spanish conquistadors (128, 129). However, an alternative explanation could be the complete replacement of a resident *H. pylori* population by a Spanish type or a selective sweep of the gene examined through the resident bacteria. Such studies of *H. pylori* genotypes could provide important clues to human ancestries and migrations.

Chapter III
Aims & Objectives

AIMS AND OBJECTIVES

Prevalence studies have indicated that *H. pylori* infection is extremely common in Bangladesh as in other developing countries. Most of our present understandings of *H. pylori* are based on studies of strains from the West and a few Asian countries. However, information on phenotypic characteristics, diagnostic methods, antimicrobial susceptibility pattern, virulence markers and genotypes of the Bangladeshi *H. pylori* strains were lacking. In this context we have characterized Bangladeshi *H. pylori* strains:

1. To study the sensitivity of stool antigen test compared to urea breath test (UBT) for the diagnosis of *H. pylori* among children and adults.
2. To study antibody responses in children against virulence proteins of *H. pylori* isolate.
3. To study the risk factor for gastric ulcer disease in patients of *H. pylori* infection.
4. To study antimicrobial susceptibility pattern of *H. pylori* isolates in Bangladesh.
5. To characterize virulence genes in *H. pylori* isolates from Bangladesh that are of potential importance in colonization or disease.
6. Comparison of genotype, in order to gain new insights into the population genetic structure of this pathogen, and to learn if genotypes implicated in the disease in the west is similarly disease associated in Bangladesh.

Chapter IV
Materials & Methods

Materials and Methods

4.1 Study site

The present study was conducted between April 1999 to 2002 at Dhaka Medical College Hospital (DMCH), Nandipara ICDDR, B study clinic and at ICDDR, B Dhaka Hospital.

4.2 Study populations and selection of patients

Objective 1:

The study was carried out at Nandipara, a periurban community 7 miles northeast of Dhaka city and populated by people of low socioeconomic status. A population of >3,000 with ~500 children <5 years of age live in a 2.5 square miles area. Among the dwellers, 75% of the males are classified as day laborers, 20% as rickshaw pullers, and 5% as carpenters, servicemen, or small businessmen. Fifteen percent of the women are day laborers and 85% are housewives. Most of the families live in poorly constructed houses with mud or thatched walls and tin or thatched roofs. The average family size is five members. The slum has a municipal waters supply for drinking and cooking, but for bathing, washing, and cleaning utensils, water from ponds and ditches is used. About 90% of the population uses hanging latrines, which are poorly constructed on bamboo poles near ponds or ditches, resulting in high fecal contamination of water. Most children defecate in open spaces. A clinic for minor illness has been run in Nandipara since 1986 by the International center for Diarrhoeal Disease Research, Bangladesh (ICDDR, B). Informed consent was obtained from the parents of the children and the Ethical Review Committee of the ICDDR, B, approved the studies.

A total of 320 randomly selected children (both gender; age 1-5 yrs) no symptomps of peptic ulcer not receive antibiotics in last month were enrolled in the study. The enrolled subjects were screened for *H. pylori* infection by UBT.Stool specimen was

collected from each children within the day of the UBT in a clean universal container for *H. pylori* Stool Antigen (HpSA) test.

Objective 2:

The virulence factors and pathogenesis, which influence the clinical outcome of *H. pylori* infections, are still poorly understood. Antibody responses in children against selected virulence proteins CagA and VacA were studied.

A total of 182 asymptomatic and apparently healthy children, aged 1-5 years, recruited from Nandipara, a peri-urban community of the Dhaka city where International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR, B) has a field clinic described previously. All children were screened for *H. pylori* infection by ^{13}C urea breath test (UBT). Children with systemic infection, severe inflammatory process or severe malnutrition were excluded. Blood samples were taken after the UBT and sera were separated by centrifugation and were stored at –20°C until assayed. Parental informed consent was required for participation of each child in the study and the study was cleared by the Ethical Review Committee of ICDDR, B.

Objective3-6:

Patients enrolled for thus part of the study were recruited from those who presented with various gastric complications to DMCH endoscopes Unit for endoscopes. Detailed demographic information such as age, sex, socioeconomic status, profession, income, smoking, alcohol consumption, or tobacco consumption habits were collected. The various symptom and history of the patients such as pain discomfort, gastrointestinal bleeding, vomiting and other symptoms were recorded before endoscopies. All person's endoscopes had provided with informed consent form according to the protocols approved by the ethical committee of DMCH and ICDDR, B. Patients on proton pump blocker, antibiotic therapy, non-steroidal anti-inflammatory drugs or antihistamine drug before the day of endoscopes was excluded.

4.3 Specimen collections and transportation

4.3.1 Materials needed for collection:

- Endoscopes machine
- Biopsy forceps
- 18gz needle
- Stuart transport media
- Normal saline
- 70% alcohol
- Sterile distilled water
- Gloves
- Plastic loop
- Biohazard Hood
- Double gas incubator
- Cidex
- Stool container
- Brain heart infusion agar (BHIA) plates

4.3.2 Preparation of Stuart transport media

Dehydrated Stuart transport media (Merck, Germany), a medium that provides a moistened, reduced oxygen environment and suppresses bacterial contamination, was used to keep the viability of the bacteria, which is susceptible to O_2 and desiccation. The medium was prepared according to the manufactories instructions (i.e. adding 19 grams of Stuart's transport medium in to 1L of distilled water).

4.3.3 Bacteriology

Collection and transportation of biopsy samples

Patients as well as healthy volunteers underwent a non-sedated upper gastrointestinal endoscopies (GIF XQ 30, Olympus Optical Company, Japan) under topical lignocaine anesthesia. Between patients the endoscopes was carefully cleaned and disinfected by first keeping the tube immersed in cidex (2.2-2.4% activated glutaraldehyde solution, Johnson and Johnson ltd) solution for 10 minutes and then rinsing it with sterile distilled water. During endoscopy two biopsy samples were taken from the antrum within 2 cm of the pylorus and two samples were taken from the body of the stomach within 8 cm of the cardia along the great curvature(111, 112). Among the two biopsies from each site, one was used for an in house rapid urease test and the other

was stored in 1ml Brain heart infusion broth containing 25% glycerol and transported to the laboratory for *H. pylori* culture. The biopsy samples were either processed immediately or stored at –86°C till use.

4.3.4 Selective isolation of *H. pylori*

4.3.4.1 Collection of defibrinated sheep blood

Sheep blood was collected by aseptically puncturing the jugular vein of a sheep and the blood was collected in a sterile wide mouthed glass bottle containing glass beads. During collection the flask was agitated in a rotary fashion for 5 minutes. The defibrinated blood was aseptically transferred into another sterile container using sterile pipette and stored at 4°C. The blood was usually used within 1 week of collection.

4.3.5 Composition of selective medium

Brain Heart Infusion Agar (BHIA) (Difco Laboratories, USA) was used as the basal medium to support the growth of *H. pylori*. BHIA media mixed with water was autoclaved at 121°C, 15lbs,for 15 minutes, and the medium was allowed to cool to 50°C in a water bath and then 35ml of warm defibrinated sheep blood was added aseptically to 465ml of the BHIA in a horizontal laminar flow working chamber followed by the addition of 0.4% isovitalex (Becton Dickinson Microbiology Systems, USA), Dent supplement (Oxoid), and tetrazolium. The antibiotic supplemented blood agar medium was mixed well and poured (~25 ml /plate) quickly and aseptically in sterile disposable plastic petri plates. For antimicrobial susceptibility test plates were prepared with different concentration of antibiotics. After solidification the plates were stored in cool and dry place, in polythene bags. Plates were used within two weeks. Prior to use the plates were dried for about1 hour at 37°C.

4.3.6 Growth conditions

Biopsies collected in the hospital were transported at 4°C into Stuart transport media and also BHIB media and immediately transferred in the laboratory. From Stuart media biopsies were transferred into BHIB and vortex for 5-6 minutes and 200µl of the resuspended was mixed with 100µl of 0.008M urea and 0.006N HCl to minimize contamination. The mixture was streaked on antibiotic supplemented blood-BHIA plates The seeded plates were incubated for 3 to 6 days at 37°C in a double gas incubator (Forma Scientific) with the concentration of gases calibrated at the following values: 5% O_2, 10% CO_2, and 85% N_2. The incubated plates were periodically examined from 2 days onwards for the growth of *H.pylori.* Presumptive *H. pylori* colonies were propagated on new plate for confirmation and stock for storage at –86°C.

4.3.7 Presumptive identification of *H. pylori*

Identification of *H. pylori* began by noting the typical colony morphology on selective blood agar medium. *H. pylori* appeared as translucent to pale grayish colonies of 0.5 to 1.0 mm in size on blood agar plates. Selective colonies were picked and sub cultured on the new plate for sufficient growth for stock.

4.4 Morphological identification

4.4.1 Gram staining

Gram staining was used for the study of bacterial morphology and gram reaction. A drop of physiological saline was taken on a clean sterile glass slide. A portion of bacterial colony was taken by a sterile loop and was suspended in the physiological saline. The suspension was spreaded homogeneously over a small area to make a thin film and the film was allowed to dry in air. The smear was then fixed by slightly heating the slide over the gas burner. The fixed smear was then flooded with crystal violet staining solution for one minute. It was flushed with tap water and again flooded with iodine solution for one minute. The slide was washed with tap water and

decolourization was done by applying 95% alcohol for 30 seconds. The slide was then washed with water and finally the smear was flooded with carbolfucsin as counter stain for 1 minute. The slide was then washed thoroughly in tap water, blotted to dry.

4.4.2 Direct microscopic observation

The slide was examined under a bright field microscope by using immersion oil.
The findings of gram-negative spiral rod shaped bacteria gave presumptive evidence of *H. pylori*.

4.5 Biochemical test

Oxidase test

Oxidase test was performed to detect the presence of cytochrome oxidase in the organism. The test was carried out by wet filter paper method (53). A strip of whatman filter No.1 was soaked with 2-3 drops of 1% tetramethyl-p-phenylene diamine dihydrochloride (Marion Scientific). A portion of the colony was picked up from the culture plate with a sterile toothpick and rubbed onto the paper. A positive reaction was indicated by formation of deep violet color.

Catalase test

This test demonstrates the presence of catalase enzyme in the organism. A small amount of culture was picked with a sterile toothpick and was dipped into 1-2 drops of 30% H_2O_2 taken on a clean glass slide. Immediate production of gas bubbles was considered as a positive result [177].

Rapid urease test (RUT)

Urea agar (Oxoid LTD, England) medium was aseptically prepared 2.4 gram was dissolved in 90 ml of distilled water and brought to dissolve completely. It was sterilized by autoclaving at 115°C for 20 minutes and cooled to 50°C. [High

temperature destroys urea so; urea solution must be sterilized by Millipore membrane filter 0.22um pore size]. Then 10.0 ml of sterile 20% urea solution was aseptically added to it and mixed well. Then aliquoted into 4 ml sterile one drum vial.

H. pylori are a more powerful producer of urease than almost any other bacterial species. Which is done by urea agar or CLO test (Delta west) strip. Urea agar strip was in occulated with pure culture and kept at room temperature for 10 minutes the colour change from yellow to pink indicates positive, or unchanged consider as negative.

4.6 Preservation of organism

Pure colonies of identified *H. pylori* were inoculated in to another plate containing 7% sheep blood with the supplements, at 37°C for 2 days under microaeroplilic condition. Pure culture was scrapped with sterile loop and stock in BHIB with 30% glycerol and kept at 86°C for long time.

4.7 Methods for diagnosing of *H. pylori* infection

There are several methods, which can be used to diagnose whether a patient is infected with *H. pylori*. They differ in being invasive or non-invasive, simple or difficult, cheap or expensive. Though far from an exhaustive coverage, the main points of each method are given below.

4.7.1 Invasive methods

Culture

H. pylori can be cultured only when a specimen containing the pathogen has been obtained, and in this case that means obtaining a biopsy specimen at endoscopes. Methods of culture vary, but in general they involve homogenizing the biopsy specimen and culturing the homogenate on a variety of specialized agar plates at elevated temperatures for at least three to six days. Culture is generally regarded as the 'gold standard' for detecting a bacterium. For *H. pylori*, however, the success of the technique depends on access to facilities, and can be regarded as being no more

than 60 - 90% sensitive, though being 100% specific; the cost of each test is high (56, 205).

Histology

Histological examination of tissue biopsy samples (usually four, taken from different parts of the stomach lining) permits detection of the bacterium together with evaluation of tissue damage. Most infection can be detected with haematoxylin & eosin (H&E) stain of gastric tissue, but special stains like Giemsa can be used if H&E results are not conclusive. The sensitivity of Wright-Giemsa and Brown-Hoops *H.pylori* stains has been shown to be 100%, compared with H&E(152), though histological detection of the organism has generally been considered to have lower sensitivity at 80 - 95% with 100% specificity. The need for a number of biopsy specimens to be examined by experienced pathologists renders histology expensive, and it requires an invasive procedure.

CLO test

The CLO test is for *Campylobacter*-like organisms. At the time of endoscopes and biopsy, a pinch of tissue is removed and placed in a test solution containing urea, a pH colour reagent and a bacteriostatic agent. The presence of urease from *H. pylori* results in hydrolysis of neutral urea to alkaline ammonia, together with a pH change and a change in colour (usually from yellow to red).

In infected tissues the change occurs within about one hour, so that the results of the CLO test are often available while the patient is still at the endoscopes unit, meaning that therapy decisions can be made immediately. Though the test itself is inexpensive, it still requires an invasive procedure to obtain the sample. It has 90 - 95% sensitivity and high specificity, and the overall cost is moderate.

4.7.2 Non-invasive method

4.7.2.1 Breath testing

Urea breath testing is non-invasive, and a specific indicator of *H. pylori* infection (15). A breath sample was collected for baseline $^{13}CO_2$ in a vacutainer following a fasting period of 2 hr. A volume of 100 ml whole milk was then allowed to be consumed by the patient. Ten minutes later [^{13}C] urea (99% ^{13}C, Tracer Technologies, Boston, Massachusetts) was given after dissolving in 25ml of water in a dose of 40 mg. After administration of the substrate, breath samples were collected after 30 min through two-way pediatric masks with an attached non-return inlet valve into a vacutainer. Samples collected in duplicate were stored for shipment to University of Basel, Switzerland, to measure the ^{13}C concentration of respiratory CO_2 by mass spectrometer. The excess over the base line value was expressed as parts per thousand (delta ‰). A breath test in which the excess over baseline was at least 5.0‰ at 30 min was regarded as positive for *H. pylori* infection.

The presence of large amounts of *H. pylori* urease results in the production of labeled CO_2, which is absorbed into the blood and excreted in the breath. Samples of breath are collected before and some time after the ingestion of labeled urea and the amount of labeled carbon dioxide measured by mass spectrometry or radioactive counting. While quite specific, few detection systems are readily available, and they do have a significant capital cost. Breath samples can be sent by post to measuring centers, though this involves a necessary delay before results are available. Breath testing is both sensitive (at least 95%) and specific (at least 98%) with a moderate cost.

Breath testing has the important advantage of being both non-invasive, and being able to confirm *H. pylori* eradication about one month or so after treatment.

4.7.2.2 Antibody measurement

Patients infected with *H. pylori* have immunoglobulin antibodies to the organism. Tests for the detection of antibodies to *H. pylori* circulating in blood, or found in saliva, have excellent sensitivity and specificity (above 95%) and are cheap and

simple compared with invasive techniques (56). They can give very quick results even within minutes of the first consultation, and are the only tests which are not likely to give false negative results in patients who have taken antibiotics, bismuth compounds or omeprazole in the recent (NIH Consensus Conference1994).

Because there are different strains of *H. pylori*, antigen for antibody detection is generally prepared by using preparations from several different strains. Antibody assays in blood have measured IgG and IgA antibodies, which have been shown to be specific for *H. pylori* and not other gram-negative organisms (205). Where both IgG and IgA assays have been compared with other testing methods, like culture and/or histology, IgG assays tend to have slightly higher sensitivity and specificity, and so anti-lgG methods tend to be favoured (51, 88, 204).

The commercially available assays are of two types: either microtitre-plate assays for use in a laboratory, or near-patient testing devices. Both types of assays usually have a cut-off value set with control sera so that they differentiate patients with *H. pylori* infection from those who do not, rather than quantify the concentration of circulating anti-*H.pylori* immunoglobulin. Laboratory based microtitre assays (51) and near-patient testing devices (209) perform equally well compared with standard techniques. Tests for antibodies to *H. pylori* in saliva are equally effective as those for antibody in serum (199). In saliva IgG immunoglobulin must be measured, as measuring IgA antibodies does not distinguish positive from negative cases. Antibody tests have high sensitivity and specificity when compared with other methods. They also have the advantage of being non-invasive, and near-patient versions are available which can produce results from a small finger prick of blood within a few minutes. The costs of antibody tests (bedside or laboratory) are much lower than any other method. Blaser et.al. Suggested that for *H. pylori* infection, serology testing may be the "gold standard" (27). Essentially, every person whose gastric biopsy can be shown to harbor *H. pylori* has evidence of a systemic hum oral immune response. Those in whom the organism cannot be detected by other methods have a low false positive rate by serology. However, gastritis can be a patchy phenomenon and histological biopsy and culture assess only a small area of the stomach, while serology in essence assays the entire stomach. "False-positive" serology may well, in fact, reflect falsely negative biopsy results.

4.7.2.3 Quantitation of antibody

Knowing how much antibody was present in the blood has not generally been thought to be helpful. Very high concentrations have been found in infected patients who also have gastric cancer (79), and more recently Nomura and colleagues (1994) found that there was a higher degree of association between *H. pylori* infection with higher levels of circulating antibody. The extent of the elevation in this study was much lower than that seen by Forman and colleagues for gastric cancer.

Almost all studies have concentrated on demonstrating the presence of antibodies to *H. pylori* as a means of diagnosis of infection. Systematic attempts to correlate levels of antibody to particular disease states have not been conducted. It is interesting to speculate that tests, which discriminate actual circulating concentrations of antibodies to *H. pylori*, might increase significantly the diagnostic yield for both peptic ulcer disease and gastric cancer, thus making screening a viable option for these diseases.

4.7.2.4 Antibody testing after *H. pylori* eradication

Titers of IgG and IgA in serum fall relatively slowly after *H. pylori* have been successfully eradicated. Thus six weeks after eradication titers fall by about 20-30%, but by six months, about 97% of patients have titers reduced by 50% or more from pre-treatment levels (138). Because of this slow reduction in antibody titers after successful eradication, serology testing is not generally useful in confirming the success of eradication within a few weeks of treatment; urea breath tests and histology or culture after repeat endoscopes can make that confirmation, if needed. However, since *H. pylori* reinfection or regrowth can take place within the first six months after treatment, waiting at least six months before retesting may be a better option.

However, there is one report, albeit of a preliminary nature, which indicates that in saliva, antibody titers to particular *H. pylori* membrane antigen fall much more rapidly (43). In a small number of patients it was shown that 83% had falls in salivary antibody titers of more than 50% within one month of successful eradication therapy. As an indicator of successful treatment, this is similar to standard methods for monitoring elimination.

4.8 ELISA for the detection of *H. pylori* antigens in stool specimens

- The *H. pylori* stool antigen test is not reliable for diagnosing *H. pylori* infection in patients with bleeding peptic ulcers.
- **Stool antigen test.** A stool antigen test detects substances that trigger the immune system to fight an *H. pylori* infection (*H. pylori* antigens). In an *H. pylori* infection, these antigens are shed in a person's feces (stool). Stool antigen testing is less expensive than the other tests and its results can be obtained quickly (in about 3 hours). Stool antigen testing may be done to help support a diagnosis of *H. pylori* infection or to determine whether treatment for an *H. pylori* infection has been successful.

Stool antigen test was done to:

- Determine whether an *H. pylori* infection may be causing a stomach ulcer, intestinal (duodenal) ulcer, or gastritis.
- Determine whether treatment for an *H. pylori* infection has been successful.

H. pylori infect the gastric mucosa and causes many digestive disorders such as peptic ulcer, chronic gastritis and gastric cancer. *H. pylori* infection relates neither to functional health status, nor to intensity of dyspepsia. There is evidence that in most patients with *H. pylori* positive functional dyspepsia do not improve with eradication of the organism. This study evaluated the diagnostic accuracy of HpSA by determining the sensitivity and specificity of the stool antigen test in predicting successful eradication during and after anti microbial therapy. The work was conducted on patients who underwent upper gastrointestinal endoscopes with dyspepsia were selected, the exclusion criteria included use of antibiotics and proton pump inhibitors up to one month before the study. All cases were submitted to, full history, general and local examination and upper gastrointestinal endoscopes. Stool samples were taken to detect *H. pylori* stool antigen. Positive patients received eradication treatment for one month and *H. pylori* status was re-determined by rapid urease test, histological examination and HpSA test one month later.

4.8.1 Materials

- Antibody Coated Micro wells (48)-brekway plastic wells coated with rabbit polyclonal antibodies specific for *H. pylori.*
- Positive Control-Inactivated *H. pylori* ATCC#43504 37.5ug protein in a pH7.2, 10mM phosphate buffered solution with 0.02% Thimerosol.
- Negative Control- pH 7.2, 10mM P BS with 0.02% Thimersol
- Sample Diluent-pH7.2, 10mMPBS with 0.02% Thimersol
- 20X Wash Buffer-pH6.8, 180mM PBS with 0.02 % Thimersol Enzyme Conjugate –Rabbit polyclonal antibody specific for *H. pylori* conjugated to horseradish peroxidase in a pH7.8, 50mM Tris buffered solution containing 0.02% Thimersol.
- Substrate-pH 5.0,100mM Citrate-acetate buffered solution containing urea peroxidase and 3,3', 5,5' tetramethylbenzidine.
- Stop Solution-2N sulfuric acid
- Transfer pipettes
- Microwell strip holder
- Microwell strip sealer
- Wooden stick applicators

4.8.2 Specimen preparation

At first 200µl of Sample Diluent was placed to a clean 12x75 mm test tube and the specimen (100 µl for liquid or semi-solid stool and 0.1g for solid stool) was diluted and mixed thoroughly vortex for several times. Formed /Solid stools-Using a wooden applicator stick, a small portion (5-6mmdiameter) of stool transferred and mixed thoroughly in to sample diluent and emulsified stool using the wooden applicator stick, then vortex 15 seconds.

4.8.3 Procedure

An enzyme immunoassay (Premier Platinum HpSA, Meridian Diagnostics Inc., Cincinnati, Ohio, USA) was used to detect *H. pylori* in the stool. It utilizes an immunoaffinity purified polyclonal anti-*H. pylori* rabbit antibody absorbed to micro wells. Fecal specimens were stored at -70°C until the test was performed. The assay was performed according to the manufacturer's instructions. Now one free falling drop of positive and negative control was placed to the specific well. Diluted fecal samples (50 µl) and a peroxidase conjugated polyclonal antibody (one free falling drop) were added to the wells and incubated for one hour at (22- 27)°C. After complete the incubation sealer was opened and washed with IX wash buffer, washing repeated for four times, to remove any unbound material, the underside of the plate was cleaned with lint- free tissue and a substrate (two-free falling drops) was added before a further 10 minutes of incubation. A stop solution (one free falling drop) was then added. The initial color of positive reaction is blue, which changes to yellow upon addition of stop solution. Visual determination and absorbance was measured spectrophotometrically both at 450 and 450/630 nm by a same person within 15 minutes after adding stop solution and recorded. The results were assigned to positive or negative on the basis of color development (for visual determination) and the manufacturer's recommended cut off values (for spectrophotometer determination).

4.9 Antimicrobial susceptibility testing

4.9.1 Agar dilution method to determine minimum inhibitory concentrations (MICs):

The lowest concentration of an antimicrobial agent that causes complete inhibition of growth under standard condition is called MIC. The MICs at which 50% and 90% of the isolates are inhibited called MIC50 and MIC90 respectively. MICs to metronidazole (mtz), clarithromycin (cla), tetracycline (tet) and amoxicillin (amx) for *H. pylori* isolates were determined by agar dilution method (98).

For each antibiotic different concentration of antibiotics were used such as, metronidazole (Sigma, St.Louis, MO) 0.05 – 64 μg/ml, tetracycline (Sigma) 0.05 – 64 μg/ml, amoxicillin 0.05 – 8 μg/ml, and clarithromycin (Abbot Laboratories, Chicago, IL,) 0.125 – 8 μg/ml.

Frozen bacterial cultures were streaked on BHI agar plate and incubated for three days. Cells from one or a few colonies from these initial plates were then re-streaked on fresh BHI agar and incubated for one more day. The resulting exponentially growing cells were suspended in PBS buffer; a series of 10-fold dilutions of these cell suspensions was prepared and 10μl of each dilution was spotted on freshly prepared BHI agar containing specified concentrations of mtz, cla, tet, amx plate. Plates were incubated in 5% O_2, 10% $C0_2$ and 85% N_2 at 37°C for 3 to 4 days The antimicrobial susceptibility was judged according to breakpoint criteria defined by National Committee for Clinical Laboratory Standards (98).

4.9.2 E-Test strip method

MIC value of each antibiotic was further checked by E test strip method. Briefly, Frozen bacterial cultures were streaked on BHI agar plate and incubated for three days. Cells from one or a few colonies from these initial plates were then re-streaked on fresh BHI agar and incubated for one more day. The resulting exponentially growing cells were suspended in PBS buffer and O. D was adjusted to 1 at 600 nm. A sterile swab stick was dipped into the cell suspension and a lawn of bacterial culture was prepared onto a blood free BHIA plate. Then the strip containing different concentration of antibiotic was aseptically placed on the plate and incubated in 5% O_2, 10% CO_2 and 85% N_2 at 37°C for 3 to 4 days (37).

4.9.3 Typing of metronidazole susceptible isolates

Jeong et al. (119) had interpreted that Mtz^S *H. pylori* were of two types: type I, requiring only inactivation of *rdxA* and type II, requiring inactivation of both *rdxA* and *frxA* to become resistant; *frxA* inactivation by itself was not sufficient to confer resistance. In the present study metronidazole susceptible isolates typed (based on

their requirement for inactivation of rdxA alone or rdxA and frxA for converting into MtzR phenotype) by transformation of susceptible isolates with plasmid pBS-rdXA-cam (*rdxA*: *cat*) and pBS-frxA-kan (*frxA::kan*). Derivatives of these Mtz^S strains with null mutant alleles of the *rdxA* and frxA nitroreductase genes were done by electroporation, using standard *rdxA*: *cat* and frxA: *aph* insertion mutant DNAs, with selection on BHI agar medium containing 15μg of chloramphenicol per ml or 20μg of kanamycin per ml. The genetic structures of transformants were checked by PCR to verify that they had resulted from allelic replacement using primers *rdxA*-F and *rdxA*-R or *frxA*-F1 and *frxA*-R1, as appropriate (119).

4.10 SDS PAGE and western blot analysis

4.10.1 Protein Extraction

For immunoblotting *H. pylori* strain DH92 (which is both *cag* and *vac* positive isolate from Bangladesh) and one *H. pylori cag* negative *vac* construct mutant isolates were used as a control. Strains were grown in Brain Heart infusion agar supplemented with 7% sheep blood, 0.4% isovitalex, Dent at 37° C for 2-3 days. Cells were harvested in phosphate buffered saline pH 7.2. Absorbance was adjusted at 600nm O.D. 1. The bacterial suspension was boiled for 20 minutes and protein concentration measured and 10μg/ml protein were separated at 12% SDS PAGE using (Biorad mini protein 11) gel electrophoresis system (BIO-RAD, USA).

4.10.2 Procedure for SDS PAGE

4.10.2.1 Gel Casting

Glass plates, spacers and the combs were wiped with 70% ethanol to remove any greasy material and were assembled for casting the gel.

A 12% SDS-polyacrylamide gel was prepared to separate the proteins of different sizes, (lower gel) by mixing the following components in screw caped tube using Pasteur pipette (table 6).

The lower gel solution was poured up to the mark leaving space for upper gel and propanol was poured and allowed for polymerization for 30 minutes. After discarding propanol and washing, upper gel was poured on and appropriate comb was inserted. After completion of polymerization of the gel, the combs were removed, the gel plates were attached to gel electrophoresis apparatus and placed in the gel tank.

Table 6: Composition of upper and lower gel components

Components	Amounts	
	Lower gel	Upper gel
Deionized water	3.35 ml	6.3 ml
30% Acryl amide	4.0 ml	1.1 ml
1.5M Tris-HCl pH8.8	2.5 ml	-
0.5M Tris-HCl pH6.8	-	2.5 ml
10% SDS	100 µl	100 µl
10%NH_4 HSO_4	60 µl	40 µl
TEMED*	12 µl	12 µl

*N, N, N', N' Tetramethylethylenediamine

4.10.2.2 Sample loading

Cell suspension of *H. pylori* 20µl with 5µl sample buffer was boiled for 8 minutes, after boiling sample was kept in ice. Wells of the gel was washed with SDS running buffer and the boiled protein samples were loaded in the wells. The gel tank was filled with running buffer to its half volume. Any air bubble trapped between the plates beneath the bottom of the gel was removed by a syringe with a bent needle. The gel was run 100volt for first 15 minutes and then voltage was lifted to 200volt.After completion of the electrophoresis the power supply was put off.

4.10.3 Materials for western blotting

- Nitrocellulose membrane
- First antibody
- Second antibody
- Tris buffered saline
- Transfer buffer
- Colour developing reagent

4.10.4 Procedure of western blot

4.10.4.1 Transfer of protein from gel to nitrocellulose membrane

The gel was then transferred from the glass plate onto the 3MM What man filter paper.

A nitrocellulose membrane was cut to the equal size of the gel. Soaked by the transfer buffer and was placed on the gel. Light pressure was given on to the membrane by the finger to remove any air bubble between the gel and the membrane.

Then the gel membrane complex was sand witched using another set of porous pad and filter paper and the plastic cassette was closed tightly.

Both the plastic cassettes were fitted in the blotting apparatus and placed in the gel tank. The tank was filled by the transfer buffer. The power was put on the protein transferring process was going on for 2 hrs at 250mA and 100 volt. An icebox was placed in the gel tank to resist excessive heat. After transfer the nitrocellulose membranes were cut into stripes and immersed in 1% skimmed milk in PBS and were kept at 4°C for over night.

4.10.4.2 Detection of protein

Following washing by 1x TBS buffer+0.5% milk for 10 minutes the membrane was incubated with first antibody (patient serum1: 40 dilution) for 90 minutes at 37°C.
After incubation the membrane was washed with 1x TBS buffer+ 0.5% milk for 3x 10 minutes each wash. The strips were then incubated with alkaline phosphates conjugated goat anti human IgG antibody dilution 1:500.The control stripe was incubated with rabbit antisera 1:10000 dilutions and secondly with rabbit antibody 1:1000 dilution in PBS.Again incubated for 90 minutes at 37°C .The membrane was again washed 3x. Finally the colour was developed by adding substrate containing ice cold methanol with 4-chloro, 1-naphthol and H_2O_2 with TBS.The presence and absence of antibody against *cagA vacA* was determined by comparing the test and

control strip. For *cagA* 128kDa and for *vacA* 90kDa was scored after visual inspection of each strip with molecular weight marker and control strains.

4.11 Chromosomal DNA extraction

4.11.1 Reagents needed

The following reagents were used for chromosomal DNA extraction:

- Solution1
- 10% SDS
- Proteinase –k solution
- 3M sodium acetate (pH 7.0)
- 99%ethanol
- TE-RNase

4.11.2 Extraction procedure

One full plate culture of *H. pylori* was suspended in 500µl of sterile 1x PBS in eppendorf tube and centrifuged at 13,000 rpm for 3 minutes. The supernatant was decanted, and pellet was thoroughly resuspended in 500µl of solution 1 and then 25µl of 10%SDS was added and incubated at 65°C for 10 minutes. After then 25µl of proteinase K (stock 5 mg/ml) was added and the tube was mixed gently and incubate at 37°C for over night. Next day equal volume of TE saturated phenol: chloroform: isoamylalcohol (25:24:1) was added and mixed by vigorous vortex. Centrifuge at 13000 rpm for 5 minutes, the upper aqueous layer was carefully transferred to another tube. This step repeated for twice. Then one tenth of 3M sodium acetate and double volume of ice cold absolute ethanol added and the eppendorf was slowly mixed so that DNA precipitated like thread, which then transferred to another tube and washed with 70% alcohol. After centrifugation at 13000 rpm for 15 minutes the pellet was dried and was dissolved in 50µl TE-RNase and was kept at room temperature for 3 hours. Finally extracted DNA was preserved at –20 °C.

4.11.3 Quantitation of DNA

The quantity of DNA in each sample was measured by (Gene Quant Pro, USA). 1 µl of DNA suspension was suspended in 99 µl of sterile deionized water in eppendorf tubes and the optical density was measured at wavelength of 260 nm and 280 nm. The readings at 260nm and 280nm allow calculations of the concentration of nucleic acid and protein in the sample respectively because at these wavelengths the absorbance of nucleic acid and protein is highest.

An optical density of 1 at 260nm corresponds to approximately 50µgm/ml double stranded DNA. The ratio between the readings at 260nm and 280nm provides an estimate of the purity of the nucleic acid. Pure preparations of DNA have OD260/OD 280 values of 1.8. If there is contamination with protein or phenol or salts the O.D 260nm/O.D.280nm will be significantly less than 1.8.

4.12 Molecular typing of *Helicobacter pylori*

4.12.1 Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) is a powerful technique that allows amplification of a specific DNA sequence millions of times in just a few hours. Dr. Kary Mulis invented the technique in 1983. During PCR, high temperature is used to separate the double stranded DNA molecule into single strand and synthetic sequences of single-stranded DNA serves as primers. Two different primer sequences are used to bracket the target region to be amplified. One primer is complimentary to one DNA strand at the beginning of the target regions and second primer is complimentary to the other strand at the end of the target region. Several parameters that can influence the outcome of PCR are taken into consideration while doing the PCR. These are

4.12.1.1 Principle of PCR

Two short single stranded oligonucleotide whose sequence is complementary to the target DNA are used as primer. These primer pair binds to the target DNA and allow initiation of DNA synthesis from target DNA, as DNA polymerases require primer to initiate DNA synthesis. By using dNTP from reaction mix Taq polymerase then synthesize a new DNA strand through a three-step process referred to as a cycle that is repeated for 30-40 cycles.

One PCR cycle consists of the following steps (Fig 4):

Step 1: Denaturation

During denaturation, the double stranded DNA is separated into two single strands by heating at 94° C. Since the hydrogen bonds linking the bases to one another are weak, they break at high temperatures, whereas the bonds between deoxyribose and phosphates are stronger covalent bonds, remain intact. This single stranded DNA then act as templates in the next round of DNA synthesis.

Step 2: Annealing-Primer binding to Target

The temperature is reduced to around 55° C to allow the primers to anneal to the target DNA.

Step 3: Extension

Once the primers anneal to the target DNA sequences, the temperature is raised to approximately 72°C and the Taq DNA polymerase begins the synthesis process at the region marked by the primers. It synthesizes new double stranded DNA molecules, both identical to the original double stranded target DNA region. The new DNA strand then serve as a template for the enzymatic synthesis of new DNA strands in the next PCR cycle by the polymerase. Each PCR cycle theoretically doubles the amount of target DNA; one cycle yield 2, then 4, then 8, then 16, then 32 copies, resulting in

exponential increases in the target which is sufficient to form a discrete band after 30-40 cycle when analyzed by agarose gel electrophoresis.

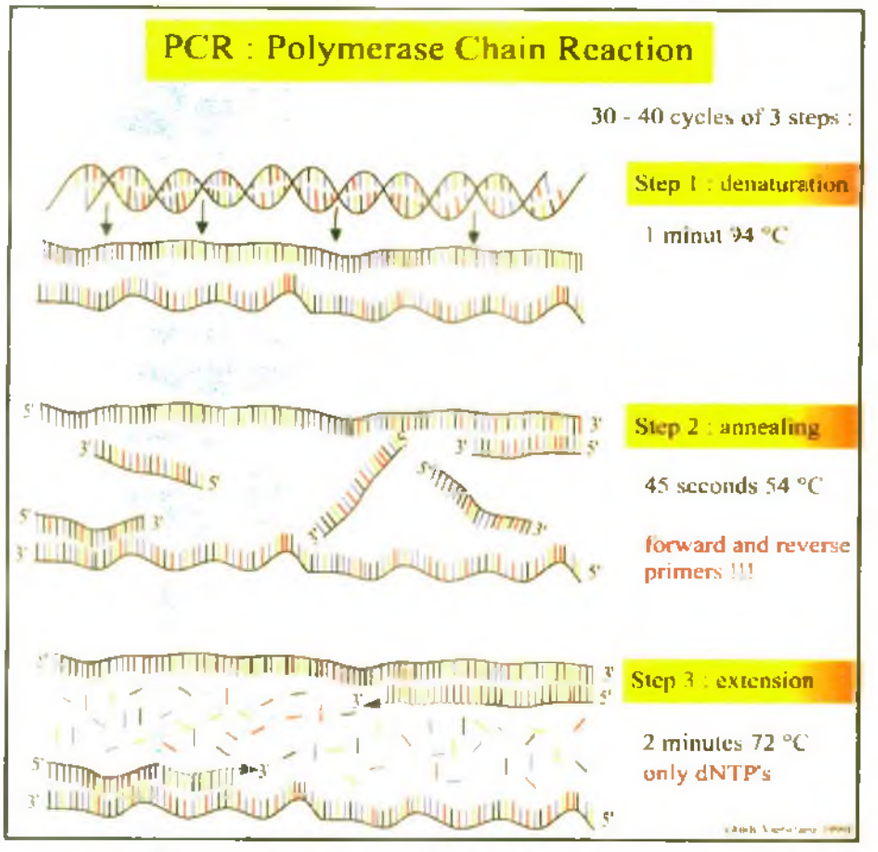


Fig 4: The different steps in PCR

4.12.1.2 Factors affecting PCR

Primer selection

The most critical parameter for a successful PCR is the designing of primer. A poorly designed primer can result in little or no product formation due to non-specific amplification or primer dimmer formation that suppresses product formation. So several parameters are taken into consideration when designing PCR primers.

These are:

Primer length

Oligonucleotides between 18-24 bases are extremely sequence specific. The more the primer length, the more inefficient binding of primer to the template DNA.

Melting temperature

Two-oligonucleotide primer should have a close melting temperature (Tm) for efficient amplification. If primers are mismatched in terms of Tm, amplification will be less efficient or may not work at all since the primer with the higher Tm will misprime at lower temperatures and the primer with the lower Tm may not work at higher temperatures. The melting temperature of primers can be calculated using the

$$Tm= \{4X (G+C)\} + \{2X (A+T)\} \ ^{o}C$$

Complimentary primer sequences

The primer sequences must be exactly complimentary to the target DNA. But the base sequences of the two primers should not be complimentary to each other. If the primer sequences are complimentary to each other then primer dimer will form which will prevent desired product formation.

G/C content

The base composition of primers should be between 45% and 55% GC. There should be no poly G or poly C stretches that promote non-specific annealing and poly A/T stretches that open-up primer template complex.

3' end sequence:

3' terminal position in PCR is essential for the control of mis-priming. Inclusion of G or C at the 3' end of primer helps to ensure correct binding at the 3' end due to stronger hydrogen bonding of G/C residues.

4.12.1.3 Component of reaction mixture

Preparation of reaction mix simply entails mixing of buffer, $MgCl_2$, dNTP, primer, Taq DNA polymerase and template. The out come PCR is influenced by the component of the reaction mixture.

$MgCl_2$

$MgCl_2$ at a particular concentration increases the activity of Taq DNA polymerase. However above a certain concentration, the activity of Taq DNA polymerase are inhibited by higher $MgCl_2$ concentration.

Deoxynucleotide tri-phosphate (dNTP)

dNTP concentration of about 200 μmole is enough to synthesize 12.5μg of DNA when half the dNTP are incorporated. dNTP chelate Mg^{2+} and thereby change the effective optimum magnesium concentration. Ultimately reduce the activity of *Taq* DNA polymerase.

Taq DNA polymerase

Taq DNA polymerase from extreme thermophile *Thermus aquaticus* is able to withstand the repeated heating and cooling steps of PCR and allow the synthesis of DNA at high temperature, which melt out the mismatched primer. Two forms of Taq DNA polymerase are now available the native enzyme purified from *Thermus aquaticus* a genetically engineered form of the enzyme synthesized in *E. coli* (AmpliTaqTm). Both forms of the polymerase carry a 5'-3' polymerization-dependent exonuclease activity, but they lack a 3'-5' exonuclease activity.

Template

For any PCR reaction plasmid DNA, chromosomal DNA or whole cell can be used as template. The two main concerns regarding to template are amount and purity of

template. If the number of contaminants are present in DNA, it van decrease the efficiency of PCR. Again the amount of template must be sufficient to be able to visualize PCR products using ethidium bromide. Usually 100ng of genomic DNA is sufficient to detect a PCR product from a single copy gene.

Primer concentration

A high primer concentration increases the probability of spurious priming and leads to the generation of nonspecific products. At the same time, it enhances the generation of primer dimmers. The concentration (C) of primer can be calculated by using the formula:

$$C\ (\text{pmole}/\mu\text{l or } \mu\text{M}) = \frac{\text{Abs. at 260nmX 100}}{1.54n_A + 0.75n_C + 1.17n_G + 0.92n_T}$$

Abs.= Absorbance at 260nm

n_A= number of adenine bases in primer

n_C= number of cytosine bases in primer

n_G= number of guanine bases in primer

n_T= number of thymine bases in primer

Cycling parameters

During each PCR cycle, the reaction mix is transferred between three temperatures. So cycling temperature should be chosen in such a way that it cause no mismatched hybrid or template primer hybridization. On the basis of GC content of primers and the length of the expected product, PCR programs are selected. Among the three steps of PCR, the annealing temperature is most important. If the temperature is too high than no hybridization takes place. If the temperature is too low then mismatched hybridization occurs. Usually the annealing temperature for a primer pair is generally calculated as 5°C lower or higher than the estimated melting temperature Tm.

4.12.1.4 Materials required for PCR reaction and Analysis

Instruments

- Thermal Cycler
- Pipettes with sterile tips box
- PCR work station or PCR Hood

Reagents

- Sterile deionized water
- 10X PCR buffer [500mM KCl, 200mM tris-Hcl (pH 8.4)]
- 2.5mM dNTP (mixture of dATP, dGTP, dCTP, dATP)
- 50mM $MgCl_2$
- 10 pmole primer 1
- 10 pmole primer 2
- Taq DNA polymerase
- Template DNA
- Agarose
- 0.5X TBE buffer
- Ethidium bromide

Table 7: Primers used in this study

Region	Primer	Nucleotide sequence
vacAs1 or *vacAs2*	VA1-F	5'-ATGGAAATACAACAAACACAC
	VA1-R	5'-CTGCTTGAATGCGCCAAAC
vac m1b	Vam-F3	5'-GGCCCCAATGCAGTCATGGAT
	Vam-R3	5'-GCTGTTAGTGCCTAAAGAAGCAT
vacA m2	VA4-F	5'-GGAGCCCCAGGAAACATTG
	VA4-R	5'-CATAACTAGCGCCTTGCAC
vacA 0.7-kb middle	VAm-F	5'-GCTCATTACGGCTTCCACTAATGT
	VAm-R	5'-GCGGTTATTGTTGTTATAAAGGGCTA
cagA (5' end)	cagA5	5'-GGCAATGGTGGTCCTGGAGCTAGGC
	cag A2	5'-GGAAATCTTTAATCTCAGTTCGG
cag PAI empty site	Luni1	5'-ACATTTTGGCTAAATAAACGCTG
	R5280	5'-GGTTGCACGCATTTTCCCTTAATC
iceA1	iceA1F	5'-TATTTCTGGAACTTGCGCAACCTGAT
	M.Hpy1R	5'-GGCCTACAACCGCATGGATAT
iceA2	cycSF	5'-CGGCTGTAGGCACTAAAGCTA
	IceA2R	5'-TCAATCCTATGTGAAACAATGATCGTT
iceA1 _94 bp	A1F673	5-GTGAGTCGTTGGGTAAGCGTTACAGAATT
	A1R1174	5'CACAACCATCATATTCAGCCTCCCCCTCATA
IS*605 orfA*	ORF18F	5'-CGCCTTGATCGTTTCAGGATTAGC
	ORF18R	5'-CAACCAACCGAAGCAAGCATAATC
IS*605 orfB*	ORF19F	5'-GGCTGTTCTAGGGTCGTGTATAAC
	ORF19R	5'-CAAGCTAGATGCAATCTAGCTACC
IS*606*	FB1	5'-GAATGTAATTCTACCTAATCCATTC
	Rb8	5'-GAGAAACCTTGATTGTTCCATG
IS*608*	608-F1	5'-CCATAACGCCTTAATAGTGTGTC
	608-R	5'-CAAGCTTTGGAGTGATGAAGTTC
Type I	cagF4856	5'-GCGATGAGAAGAATATCTTTAGCG
	cagR5280	5'-GGTTGCACGCATTTTCCCTTAATC
Type II	IS606-1692	5'-CTAACAATTTGCCATTATGCTGT
	cagF4856	5'-GCGATGAGAAGAATATCTTTAGCG
Type III	fcn unk	5'-TGGATTAAATCTTAATGAATTATCG
	cagF4856	5'-GCGATGAGAAGAATATCTTTAGCG

4.12.1.5 Procedure

Specific PCR was carried out in 50µl volume and master mix was prepared according to the composition described in table 8.

Table 8: Composition of Reaction mixture

Components	Final concentration	Volume/ reaction (µl)
10X PCR buffer	1X	5.0
50mM $MgCl_2$	1.5mM	1.5
2.5mM dNTP	25 µM	0.25
Primer 1(25pmole)	25pmole	1.0
Primer 2 (25pmole)	25pmole	1.0
Taq polymerase	1U	0.2
D/W H_2O	-	38.05
Template	180ng	3.0

The PCR was then carried out in a thermal cycler 9600 under following condition:

Initial denaturation at 95°C for 1min
at 95°C for 1min
at 52°C for 1min } 35cycles
at 72°C for 1min
Final extension at 72°C for 10min

4.12.1.6 Study of the PCR product

After completion of the desired cycle the amplified product was studied by agarose gel electrophoresis to gain information about the DNA molecule that acted as the original template.

Agarose gel electrophoresis:

A portion of the amplified reaction mix (6µl) are loaded in an 1% agarose gel and run at 100v for 1h.The loaded DNA molecule are separated through the gel according to their molecular weight and charge. The low molecular weight DNA molecules come out from the gel first whereas the high molecular weight DNA comes later. A band representing the amplified DNA is visualized after staining the gel by ethidium bromide.

Preparation of gel: 1g of agarose powder are dissolved in 100ml of 0.5x TBE (Tris Borate and EDTA buffer) buffer and 6µl ethidium bromide are added in gel to visualize the product .

4.13 Sequencing:

4.13.1 DNA Sequencing

The process of determining the order of the nucleotide bases along a DNA strand is called sequencing. In 1977, twenty-four years after the discovery of the structure of DNA, two separate methods for sequencing DNA were developed: the chain termination method (Sanger method) and the chemical degradation (Maxam Gilbert) method which used X-Ray machine to detect the location of the nucleotides. But now a days different genetic analyzer have been discovered which detect the nucleotides from fluorescence labeled DNA by laser beam instead of using X-Ray machine.

4.13.2 Principle of Sanger method

This method was devised by Sanger, hence called Sanger method. This technique utilizes 2', 3'-dideoxynucleotide triphospates (ddNTPs), molecules that differ from deoxynucleotides by the having a hydrogen atom attached to the 3' carbon rather than

an OH group. These molecules terminate DNA chain elongation because they cannot form a phosphodiester bond with the next deoxynucleotide.

In order to perform the sequencing, one must first convert double stranded DNA into single stranded DNA. This can be done by denaturing the double stranded DNA with NaOH. A Sanger reaction consists of the following: a strand to be sequenced (one of the single strands which was denatured using NaOH), DNA primers (short pieces of DNA that are both complementary to the strand which is to be sequenced and radioactively labeled at the 5' end), a mixture of a particular ddNTP (such as ddATP) with its normal dNTP (dATP in this case), and the other three dNTPs (dCTP, dGTP, and dTTP). The concentration of ddATP should be 1% of the concentration of dATP. The logic behind this ratio is that after DNA polymerase is added, the polymerization will take place and will terminate whenever a ddATP is incorporated into the growing strand. If the ddATP is only 1% of the total concentration of dip, a whole series of labeled strands will result Note that the lengths of these strands are dependent on the location of the base relative to the 5' end.

This reaction is performed four times using a different ddNTP for each reaction. When these reactions are completed, a polyacrylamide gel electrophoresis (PAGE) is performed. One reaction is loaded into one lane for a total of four lanes the gel is transferred to a nitrocellulose filter and autoradiography is performed so that only the bands with the radioactive label on the 5' end will appear. In PAGE, the shortest fragments will migrate the farthest. Therefore, the bottom-most band indicates that its particular dideoxynucleotide was added first to the labeled primer.

4.13.3 Automated DNA sequencing

In automated DNA sequencing a cycle sequencing kit is used (e.g. Big Dye^{TM} terminator cycle sequencing ready reaction kits), that contains AmpliTaq DNA polymerase and both dNTP and dye labeled ddNTP, and uses dye terminator cycle sequencing strategies. In this method only one strand of the desired DNA are copied with one primer and results in the linear increase of the number of copies of one strand of the gene. The growing chains are terminated when dye labeled terminators

(ddATP, ddGTP, ddCTP, ddTTP) are incorporated as dideoxyribonucleotides has no hydroxyl group at the 3' end which forms the phosphodiester bond with the next nucleotide. After cycle sequences unincorporated chain terminators are removed from the reaction mix with alcohol precipitation and the DNA fragments of different length, which ended on a fluorescent-labeled ddNTP, are separated according to their length through capillary electrophoresis. Finally the nucleotide bases are detected in ABI Prism-310 Genetic Analyzer machine by the laser detection system.

There are four major preparatory steps for DNA sequencing
(1) Preparation of target DNA
(2) Cycle Sequencing
(3) Purification of the cycle sequence product
(4) Sequencing

4.13.4 Preparation of target DNA

The target DNA was prepared by PCR that was described in section 4.13.4.1 The PCR product was purified by using commercially available column (MicroconYM-100). Briefly, PCR product was poured on a microfuge tube-containing sieve. The tube was centrifuged at 500g for 12 minute. Then the sieve was removed from the previous tube and placed on a new tube as upside down. Then again centrifuged at 1000g for 3 minutes. The sieve was discarded and the filtrate was used as purified template for sequencing. Then the DNA was quantitated in Gene quant pro as described in section 4.11.3.

4.13.4.1 Cycle sequencing

In cycle sequencing the ABI–prism Big Dye terminator cycle sequencing ready reaction kits is used which contain all the required components for the sequencing reaction (dye terminators, deoxynucleoside triphosphates, ampliTaq DNA polymerase, magnesium chloride and buffer). In this step only one strand of the desired DNA is amplified and the DNA fragments of different length, which has been terminated with different ddNTP, are produced.

Table 9: Template

Template	Quantity
PCR Product	
100-200bp	1-3 ng
200-500bp	3-10
500-1000bp	5-20ng
1000-2000bp	10-40ng
>2000bp	20-50 ng
Single stranded	25-50 ng
Double stranded	150-300 ng
Cosmid Bac.	0.5-1 µg
Bacterial genomic DNA	2-3 µg

Table: 10 Reaction mixtures

Reagent	Quantity
Terminator Ready Reaction Mix	4.0µl
Template	quantity on table 9
Primer	3.2 pmol
Deionized water	to make 20 µl
Total volume	20µl

Programme of cycle sequencing:

96°C	1 min	
96°C	10sec	25 cycles
50°C	5sec	
60°C	4 min	
60°C	1min	

4.13.4.2 Purification of the Extension Products

1.5 ml micro centrifuge tube containing 2.0µl of 3M sodium acetate pH 4.6 and 50.0 µl of 95% alcohol was taken. Then the entire contents of each reaction were added into a tube of sodium acetate and ethanol mixture. It was mixed thoroughly and kept at room temperature for at least 15 minutes to precipitate the extension products (precipitation Times<15 min. will result in the loss of very short extension products. Again precipitation Times >24 hours will increase the ppt. of unincorporated dye terminators). It was centrifuged for 20 min at 14000rpm and the supernatant was aspirated. The pellet was washed with 250µl of 70% alcohol, by washing the wall of the tubes. Again centrifuged for 10 min and the washing steps Repeated twice. The pellet was dried with Para film over the tube makes hole and left for over night at room temp.

4.13.4.3 Sequencing of PCR product

Dried product was dissolved in 12-15µl of template suppression buffer denature for 2min in the PCR machine and transferred in the septa tube and placed on a 48- well tray. The tray was loaded on an ABI PRISM 310 automated sequencer (PE Applied Biosystems, USA) and samples injected in to a 47cmx50 u capillary coated with POP-6 (Performance Optimized polymer-6, PE Applied Biosystems, USA) polymer using electrokinetic injection at 320 volts/cm (15 KV for the 47 cm capillary, current at this voltage 5-8 µA) and electrophoresed using the Seq POP RAPID E run module with the DT POP6 {BD Set-any primer} mobility file. The sample fluorescent spectra during electrophoresis was processed into base sequence data using the ABI PRISM 310 Data Collection Software and the raw sequencing data was analyzed using the ABI PRISM DNA Sequencing Analysis Software (PE Applied Biosystems, USA).

4.13.4.4 Assembly of relevant sequences from GenBank

To assembly the sequences of different genes of *H. pylori* strains from other geographic regions, the Basic Local Alignment Search Tool (BLAST Version 2.0) was used to search the public domain nucleotide database maintained by the national

Centre for Biological Information (NCBI, www.ncbi.nlm.nih.gov). The default parameters set for the alignment were: gapped setting, non-redundant databases, expectation value of 10, filtering of low-complexity regions by the SEG algorithm, BLOSUM 62(gap existence cost=11,per residue gap cost=1, lambda ratio=0.85) substitution matrix.

4.13.4.5 Nucleic acid alignments

The consensus nucleotide sequence of a gene segment was aligned with other known related sequences using the software program CLUSTAL-X Version 1.8 (National Center for Biological Information, USA). The gapped alignments were performed using the following parameter: Gap opening =10. gap extension =0.20, delay divergent sequences =30%, DNA transition weight=0.50.The weight matrices used were IUB for DNA.

4.13.4.6 Phylogenetic analysis

Nucleotide sequences were analyzed for the nucleotide substitution pattern in order to chose an appropriate evolutionary model for the subsequent phylogenetic analysis .All the sequences obtained were aligned with different related sequences of strains isolated from different geographical regions. Multiple alignments were done by PHYLIP program. Raw sequences were edited by Chromas, DNAsis, and Sequencher program. Phylogenetic tree was constructed from the DNA distance matrix by neighbor-joining method using NEIGHBOR available in PHYLIP package. Finally phylogenetic tree was viewed in TREE VIEW software.

Chapter V
Results

Study I

Diagnostic approach: *Helicobacter pylori* stool antigen (HpSA) test

5.1 Demographic characteristics of the enrolled children

A total of 320 randomly selected asymptomatic children from Nandipara were enrolled for stool antigen test. The demographic behavior of the children were analyzed that are described in(table 11).

Table 11: Demographic Characteristics of the study children (n=320) for HpSA vs. UBT test

Characteristics	Mean ± SD	Range
Age (Month)	38.9 ± 14.5	18-60
Weight (Kg)	11.6 ± 2.5	6.9-18.9
Height (cm)	88.9 ± 10.7	69.5-114.3
Weight for age (%)	78.6 ± 9.7	60.0-134.0
Weight for height (%)	89.9 ± 8.4	63.134.0

5.1.2 Stool antigen test (HpSA) and UBT test

Among the enrolled children 196 (320/196) were positive for *H. pylori* by UBT test and 153 (153/320) were positive for *H. pylori* by stool antigen test.

5.1.2.1 Comparison of HpSA with Urea breath test (UBT) in detection of *H. pylori* infection

Urea breath test of 320 children were done in university of Basel and the results were compared with HpSA results to determine sensitivity of the two methods for diagnosis of *H. pylori* infection. While comparing the data between UBT and HpSA, 61% children were positive for UBT and 48 % for and HpSA. Whereas 52% and 38% were negative for HpSA and UBT (Table12).

Table12: Comparison between UBT and HpSA test of the study children (n=320)

	UBT (%)	HpSA (%)
Positive	196(61.25)	153(48.96)
Negative	124(38.75)	167(52.18)

5.1.2.2 Diagnostic accuracy of *H. pylori* stool Antigen (HpSA) against UBT in the detection of *H. pylori* infection

Detection of *H. pylori* infection for diagnostic accuracy sensitivity, specificity of HpSA test was compared with gold standard UBT and was 78% and 99% respectively. The positive predictive value and negative predictive value of HpSA compared to UBT were 99%, 76% respectively. Accuracy in both the test method was 80% (Table 13).

Table13: Diagnostic accuracy of *H. pylori* stool Antigen (HpSA) against UBT

Diagnostic accuracy	Percentage	95% c.i.
Sensitivity	78	73.4-82.6
Specificity	99	97.9-100
Positive predictive value	99	97.9-100
Negative predictive value	76	71.3-80.8
Accuracy	80	75.5-84.4

Study II

High prevalence of *cagA* and *vacA* seropositivity in Bangladeshi Children with *Helicobacter pylori* infection

5.2 Western blot analysis for detection of *cagA*, *vacA* antibodies

5.2.1 Demographic Characteristics of the enrolled children

A total of 182 children with symptoms of gastric infections were enrolled in the study. UBT was done for diagnosis of *H. pylori* infection and serum sample was analyzed for antibodies against *cagA* and *vacA*. The sociodemographic characteristics of the children are shown in table 14. The mean age of the children was 43.6 month (17-60).

Among 182 children 79% were positive for *H. pylori* infection in UBT.

Table 14: Demographic characteristics of the study children (n=182)

Characteristic	Mean±SD	Range
Age (month)	43.6 ±12.6	17-60
M/F	90/92	
Weight (kg)	12.0 ±2.4	6.5-18.7
Height (cm)	90.0 ±10.0	67-114
Weight for age (%)*	77 ± 9	62-109
Weight for Height (%)*	90± 8	72-91
H. pylori status (UBT+)	(79.7%)	

*National Centre of Health Statistics (NCHS) median

5.2.2 Seroprevalence of *cagA* and *vacA* antibodies by western blot and UBT

The presence of *cagA* and *vacA* antibodies in the serum of study patients were analyzed by western blot and a combination of presence or absence of *cagA* and *vacA* were found. 82% patients had antibody against *cagA*, 81% against both *cagA* and *vacA* and 82% against *vacA* (Table 15).

In UBT positive patients 95% had antibody against *cagA* with or without *vacA*, 94% had antibody against both *cagA* and *vacA*, 97% had antibody against *vacA* with or without *cagA*.

Table 15: Seroprevalence of *CagA* and *VacA* antibodies among study children

Antibody	All study Children (n=182)	Children with positive UBT (n=145)
	Number (%)	Number (%)
CagA with or without *VacA*	149 (82)	138 (95)
CagA alone	1 (0.6)	1 (0.7)
VacA with or without *CagA*	150 (82)	140 (97)
VacA alone	2 (1)	2 (1.3)
Both *CagA* and *VacA*	148 (81)	136 (94)

5.2.3 Comparison and Agreement between Urea Breath Test and Western Blotting in Diagnosis of *H. pylori* infect ions in Children

UBT and western blot were compared it was found that out of 182 patients, 137 patients were positive for *H. pylori* in both UBT and western blot and 25 patients were negative in both methods. Among the rest 8 patients were negative in WB but positive in UBT. Where as 12 patients were negative in UBT and positive in WB (Table 16).

Table16: Comparison and Agreement between Urea Breath Test and Western Blotting in Diagnosis of *H. pylori* infections in children

Western Blotting	UBT		Total	P Value	95% c.i.
	Positive	Negative			
Positive	137	12	149		
Negative	8	25	33	0.53	0.12 to 0.78
Total	145	37	182		

Diagnostic accuracy of Western blot was further compared with UBT as gold standard the sensitivity, specificity and accuracy for Western blot were 94%, 68% and 89% respectively (Table 17).

Table17: Diagnostic accuracy of Western blotting (WB) method against Urea Breath Test (UBT) test in the detection of *H.pylori* infection

Sensitivity	94%
Specificity	68%
Positive predictive value	92%
Negative predictive value	76%
Accuracy	89%

Study III

Phenotypic and Demographic characteristics of *Helicobacter pylori* isolates in relation to disease presence

5.3 Demographic characteristics

A total of 278 consecutive patients attended at the Dhaka Medical College Endoscopy Unit during 1999 to 2002 were enrolled in this study. The age limit of the patients was 15 and 78 years and among them, 72.7% (202 patients) were male and 27.3% (76 patients) were female (Table 18).

Among the patients, 162 had PU and 116 had NUD (which was diagnosed on the basis of endoscopies examination and histological examination) and 62.6% (174 of 278) were culture positive for *H. pylori*. Among the culture positive patients, 121 (69.5%) were male and 53 (30.4%) were female and 112 (64.3%) had PU and 62 (35.6%) had NUD.

Table 18: Demographic characteristics of the study patients (n=278)

Characteristics	Range/Ratio
Age (years)	15-78 give mean age
Male/Female	202/76

5.3.1 Different risk factors with relation to Incidence of *Helicobacter pylori*

The behaviour of the patients such as smoking, alcohol consumption family history of peptic ulcer disease among patients with PUD and NUD was compared and are shown in Table 19. No significant relation between smoking habit and types of disease was observed among enrolled study subjects. 50% and 57% patients had smoking habit in PUD and NUD group respectively.

98% and 94% of the subjects in PUD and NUD group had no history of alcohol consumption.

While determining the relationship between family history and disease presence, 40% and 33% of the enrolled subjects in PUD and NUD have had history of peptic ulcer diseases among family members.

23%, 57% and 19% subjects in PUD group reported that the gastric pain were aggravated, relieved and unrelated with food consumption respectively. While in NUD patients 20%, 40%, 40% were aggravated relieved, unrelated respectively in NUD patients.

There was no clinical significance for prevalence of *H. pylori* infection with specific behavior.

Table 19: Percentage of risk factors with disease relationship

Disease (n)	Smoking Habit (%) of all patients		Alcohol consumption (%)		Family history (%)		Food habit (%)		
	Yes	No	Yes	No	Yes	No	Aggravated	Relieved	Unrelated
PUD (162)	82 (50)	80 (49)	3 (2)	159 (98)	65 (40)	97 (60)	38 (23)	93 (57)	31 (19)
NUD (116)	49 (42)	67 (57)	7 (6)	109 (94)	38 (33)	78 (67)	22 (20)	47 (40)	47 (40)

5.3.2 Phenotypic characteristics

A total of 278 patients biopsies were collected from antrum and body of the stomach were cultured, and *H. pylori* was isolated from 174 subjects. Of the 174 cultures positive isolates 33% (57), 42% (73) and 25% (44) were isolated from antrum, body, and both sites respectively (Fig 6, showed pure culture of *H.pylori*).

Relationship between site of isolation and disease presence were further studied. In case of PUD disease 68% (39/57), 68% (50/73), and 77% (34/44) isolates were isolated from antrum, body, and from both sites respectively; whereas 32% (18/57), 32% (23/73), and 23% (10/44) isolates were found in antrum, body, and both sites, respectively in case of NUD patients (Table 20).

Table 20: Culture positivity (n=174/278) on the basis of the site of biopsy collection

Site of Isolation	PUD	NUD
Antrum 33%(57)	68% (39)	32 %(18)
Body 42% (73)	68% (50)	32% (23)
Antrum and body 25% (44)	77% (34)	23% (10)

5.3.3 Rapid urease test (RUT)

All the biopsy tissues collected were tested for RUT, for the detection of *H. pylori*. Samples positive for RUT turned pink colour within 20 minutes, sometimes delayed. Samples, which were positive within 2 hours, were recorded as positive. 88% RUT positive were culture positive (Fig 5).

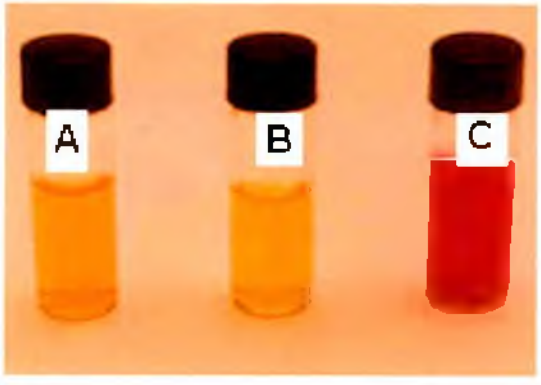


Fig 5: Photograph showing rapid urease test (RUT) using endoscopic biopsy tissues. (A) Urea agar base medium. (B) Biopsy containing urea agar base medium. (C) After 20 minutes, medium changes to pink color, it indicates the presences of *H. pylori* in biopsy samples.

5.3.4 Cultural characteristics

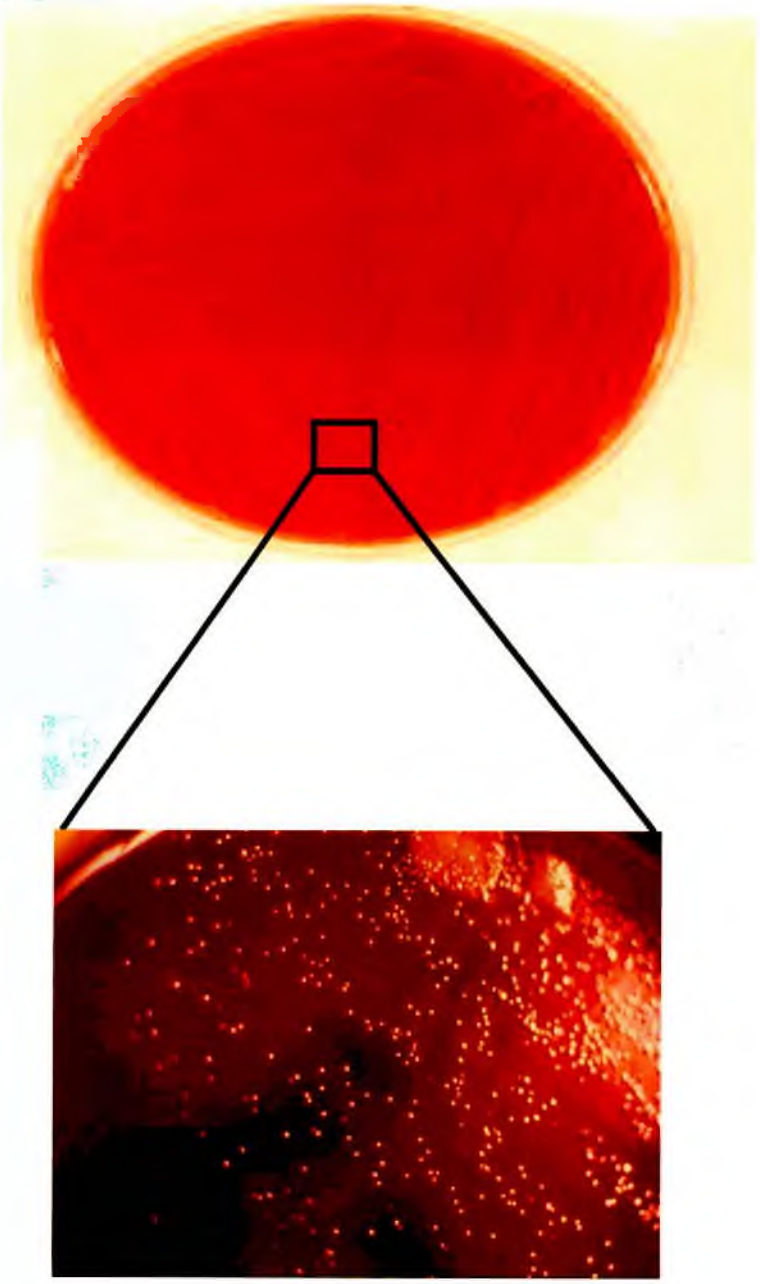


Fig 6: Pure culture of *H. pylori* on BHIB plate

5.3.5 Oxidase test

All of the 174 isolates were positive for oxidase test produced purple color when reagent reagent (N, N, N', N', tetra methyl-p- phenylenediamine dihydrochloride) was added on the filter paper and colony of *Helicobacter* was added with toothpick.

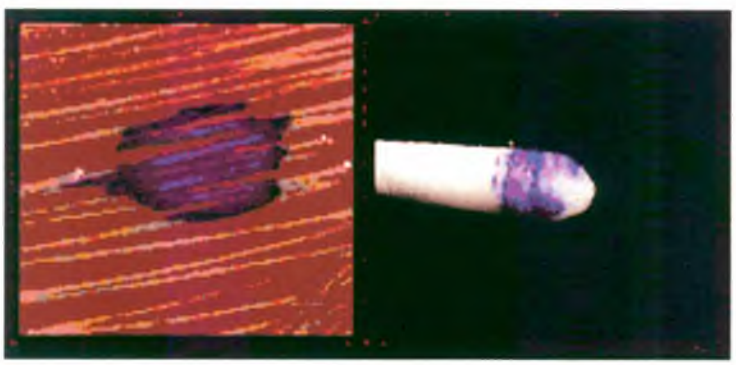


Fig 7: Positive oxidase reaction

5.3.6 Catalase test

All of the 174 isolates were positive for catalase test produced bubble on the culture when 30% H_2O_2 was added (Fig 8).

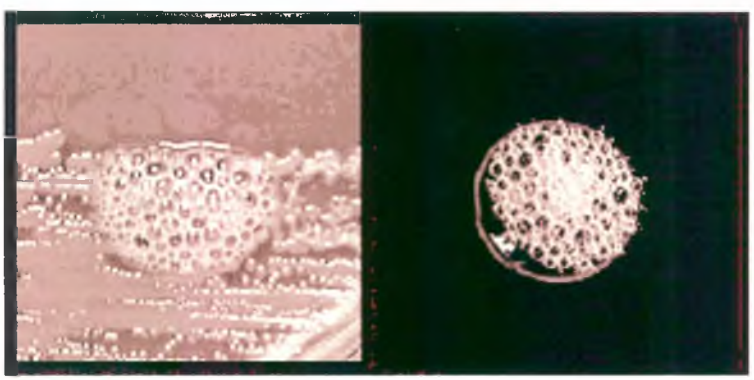


Fig 8: Positive Catalase reaction.

5.3.7 Gram stain

All of the 174 isolates were gram negative, spiral shaped under microscope (Fig 9).

Fig 9: Microscopic photograph showing gram-negative spiral shaped *H. pylori* in biopsy tissues

5.4 Antimicrobial susceptibility Testing

5.4.1 Agar dilution method to determine Minimum Inhibitory Concentrations (MICs)

Of the 174 culture positive isolates 120 were available for AMR testing. AMR testing was done by agar dilution and E test method. Of these 120 isolates 88 (73.3%) patients were peptic ulcer, 32(26.6%) patients were non-ulcer dyspepsia. Overall around 10%, 15%, 77%, 6% isolates were resistance to clarithromycin, tetracycline metronidazole, and amoxicillin respectively. In peptic ulcer patients 8%, 9% 76%, and 13% isolates were resistance to amoxicillin, clarithromycin, metronidazole, and tetracycline respectively. In NUD 3%, 12%, 81%, and 18% isolates were resistance to amoxicillin, clarithromycin, metronidazole, and tetracycline respectively. However, there was no significance between NUD and PUD patients. In male patients 4%, 8%, 77%, and 16% isolates were resistance to amoxicillin, clarithromycin, metrinidazole, and tetracycline respectively. Of the 32 female patients 12%, 15%, 78%, and 12%, isolates were resistance to amoxicillin, clarithrimycin, metronidazole, tetracycline respectively. However, there was no significance between male and female patients. (Table 21).

Table 21: Antibiotic susceptibility pattern of 120 *H. pylori* isolates (during 1999-2001) from Dhaka, Bangladesh

Antimicrobial Agents	No (%) of resistant isolates all patients (n=120	No (%) of resistant isolates PU patients (n=88)	No (%) of resistant isolates NUD patients (n=32)	No (%) of resistant isolates male patients (n=88)	No (%) of resistant isolates female patients (n=32)
Amoxicillin	8 (6.6)	7 (8.0)	1 (3.1)	4 (4.5)	4 (12.5)
Clarithromycin	12 (10)	8 (9.1)	4 (12.5)	7 (8.0)	5 (15.6)
Metronidazole	93 (77.5)	67 (76.1)	26 (81.2)	68 (77.3)	25 (78.1)
Tetracycline	18 (15)	12 (13.6)	6 (18.7)	14 (16.0)	4 (12.5)

The MICs at which 50% and 90% of the isolates tested were inhibited (MIC_{50} and MIC_{90} respectively) was further calculated for metronidazole, clarithromycin, amoxicillin and tetracycline.

MIC_{50} for metronidazole, tetracycline, clarithromycin, amoxicillin were 64, 2, 1, 1 μg/ml, respectively; and MIC_{90} for Met, Tet, Cla, Amox were 16, 0.5, 0.05, and 5 μg/ml respectively (Table22).

Table 22: MIC_{50} and MIC_{90} (μg/ml) of four antibiotics for 120 *H.pylori* isolates (during 1999-2001) from Dhaka, Bangladesh

Agent	MIC_{50}	MIC_{90}	Range
Metronidazole	64.0	16.0	0.5 - 128.0
Tetracycline	2.0	0.5	0.125 - 4.0
Clarithromycin	1.0	0.05	0.125 - 4.0
Amoxicillin	1.0	0.5	0.125 - 4.0

Study IV

Antimicrobial susceptibility pattern of *Helicobacter pylori* isolates in Bangladesh

Figure 1. max. 4 strips used on a 150 mm and only 1 on a 90 mm plate

Figure 2. Very susceptible strain where the ellipse does not intersect the strip MIC <0.016 ~µg/ml

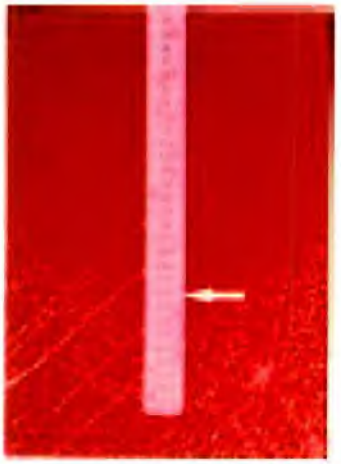


Figure 3. Weak growth and sparse inoculum. The test should be repeated if necessary (MIC 0.032 ~ µg/ml.)

Figure 4. Sharp end point. MIC 0.125 ~ µg/ml

Figure 5. High resistance to metronidazole MIC >32 µg/ml

Figure 6. Colonies of a resistant sub-population in the ellipse MIC >32 ~ µg/ml

Figure 7. Trailing of microcolonies at the end point MIC 0.5 ~ µg/ml

Figure 8. Microcolonies at the end point MIC 0.5 µg/ml

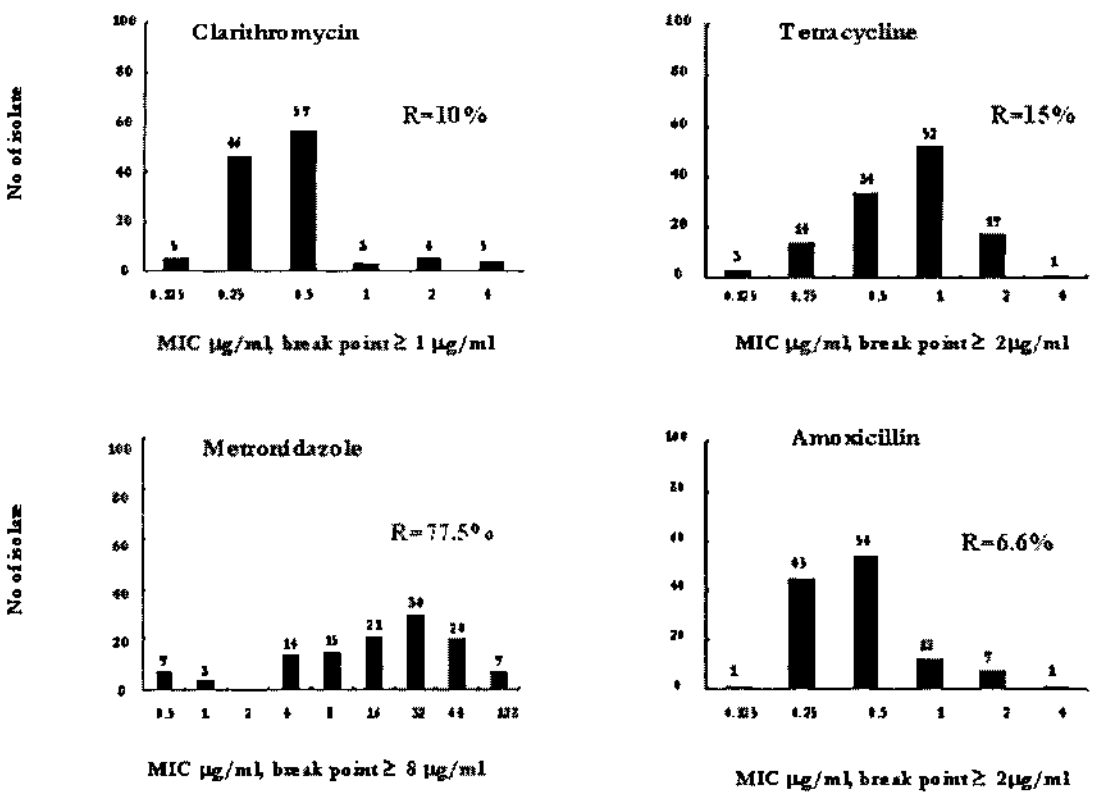
Figure 9. Contamination at the top and side of the strip MIC 1.5 µg/ml

Figure 10. E-test reading guide for *H. pylori*

The distribution of MIC for metronidazole, clarithromycin, amoxicillin and tetracycline of the isolates are shown in Fig 5.11.

It was found that 5 isolates were resistant at 4μg/ml concentration for clarithromycin, 52 isolates resistant at 1μg/ml concentration for tetracycline, 30 isolates at 32 μg/ml for metronidazole and for amoxycillin 12 isolates were resistant at 1μg/ml concentration. That is for clarithromycin 10%, tetracycline 15%, amoxicillin 6%, metronidazole 78% isolates were resistance.

Fig.11 Distribution of MIC among the tested 120 *H. pylori* isolates



Study V & VI

DNA-Level Characterization of *Helicobacter pylori* Strains from Patients with Overt Disease and with Benign Infections in Bangladesh

5.5 Genotype

5.5.1 Distribution of cag pathogenicity island (cagPAI) among Bangladeshi isolates

A total number of 174 isolates were studied for genotypic analysis. The presence of the cag PAI or of the empty site that is characteristic of cag negative strains was scored by PCR with specific primers, using DNA extracted from cultured strains. A 280 bp products indicative of cagPAI were obtained using primers specific for the *cagA* gene from 85 of 112 strains with peptic ulcer disease, and 34 of 62 from patient's non-ulcer dyspepsia. Thirty four of the cultures from which no *cagA* gene-specific PCR product was obtained an empty –site product of 550bp, in which 18 of them were peptic ulcer disease and 16 of them having non-ulcer disease. Twenty one isolates 9 from peptic ulcer disease and 12 from non ulcer disease, having specific *cagA* gene and also empty site, indicating that they had mixed infection i.e. a mixture of strains with or without the *cagA* gene (Table 23).

Table 23: Distribution of cagA in *H. pylori* from Bangladesh

Patient Clinical status	cagA		
	cagA+	cagA-	mixed
PUD (n=112)	85	18	9
NUD (n=62)	34	16	12

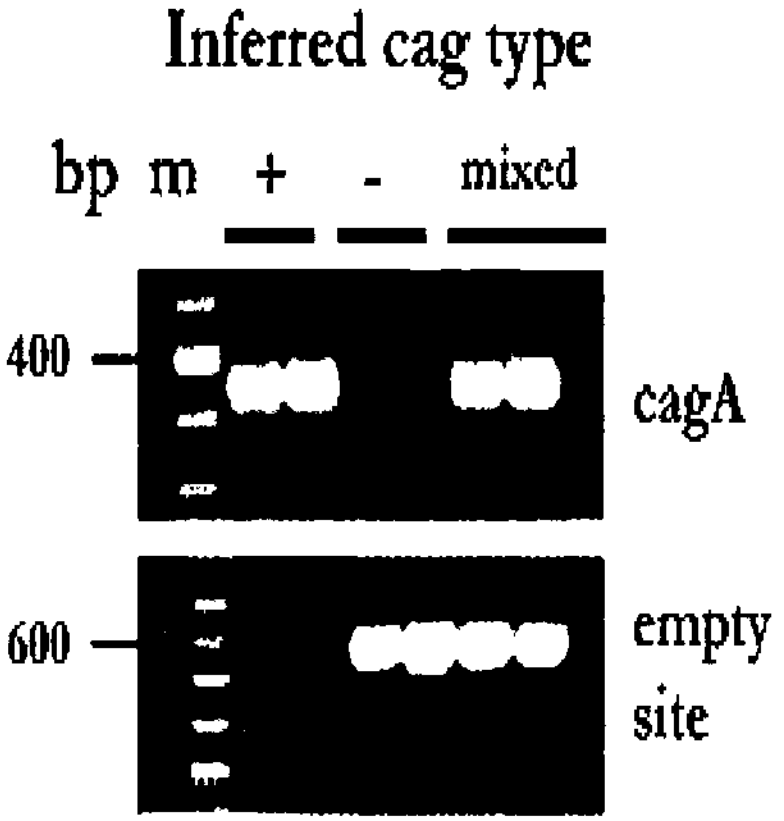


Fig12: Agarose gel electrophoresis shows PCR amplified product of CagA and empty site product

5.5.1.1 Cag right junction motifs

All isolates were tested for Cag right junction motif using type specific primers. 113 isolates belonged to Cag type I including 70.5% (79/112) in PUD group and 54.8% (34/62) in NUD group. 40 isolates belonged to Cag type II including18.75% (21/112) in PUD group and 30.64% (19/62) in NUD group.21 isolates belonged to Cag III including10.7% (12/112) in PUD group and 14.5% (9/62) in NUD group. The type III motif at the extreme right end of *cag* PAI, characterized by the presence of a function-unknown sequence replacing the *hel* gene fragment, was found in 21 of the strains were cag PAI-positive Bangladeshi strains as determined by PCR (Fig 12) (table 24).

Table 24: Distribution of cag right junction motifs *H.pylori* strains from Bangladesh

Patient Clinical status	**Cag right junction motifs**		
	Cag type I	**Cag type II**	**Cag type III**
PUD (n=112)	79	21	12
NUD (n=62)	34	19	9

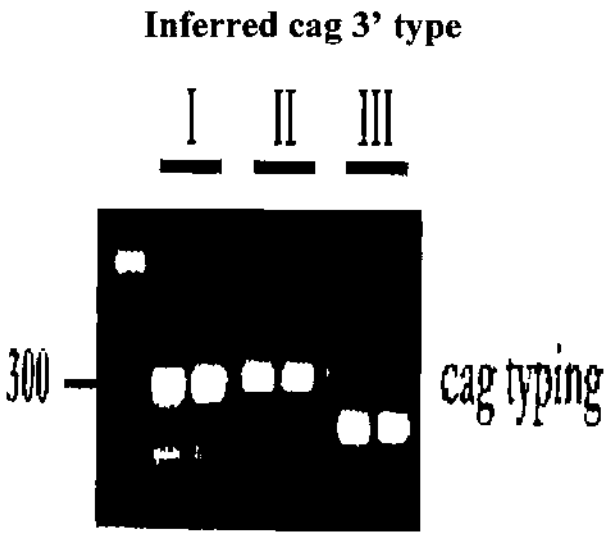


Fig13: Agarose gel electrophoresis showing PCR typing of isolates by 3' end of cag gene

5.5.2 Prevalence of vacA alleles

The presence of the s1 verses s2 alleles at the 5' end of the vacuolating cytotoxin (vacA) gene was scored based on the sizes of PCR products generated with specific primers. 81.25% (91/112) of isolates from patients with PUD 33.03% (37/112) of isolates from patients with NUD yielded only 259 bp fragment indicating s1 allele; 18 of the peptic ulcer and 19 of non ulcer disease patients yielded 286bp fragment indicating s2 allele; and 3 of PUD, 6from NUD patients yielded 259 and 286bp fragment indicating an s1 and s2 mixed infection (Table 25).

Table25: Distribution of vacA s-alleles in *H. pylori* strains from Bangladesh

Patient Clinical status	vacA s-alleles		
	s1	s2	s1s2
PUD (n=112)	91	18	3
NUD (n=62)	37	19	6

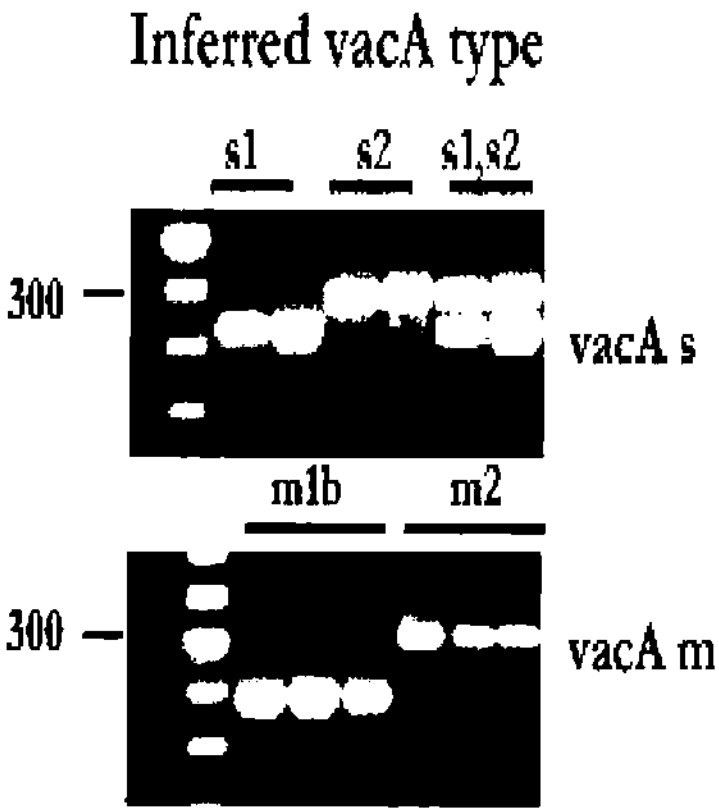


Fig14: Agarose gel electrophoresis showing PCR amplified product of vacA allele and vacA middle region allele

5.5.3 Distribution of vacA m-alleles

The prevalence of the alleles of middle (m) region of *vacA*, a part that affects host specificity, was also determined by PCR. Products were obtained only with *vacAm1b* primers in strains of 67 patients with PUD and 25 patients with NUD (Table26), whereas PCR amplification with only *vacAm2* primers occurred in the cases of 39 of 112 patients with PU and 25 of 62 patients with NUD. Both m1b and m2 products were observed in six patients with PU and twelve patients with NUD.

Table 26: Distribution of vacA m-alleles in *H. pylori* strains from Bangladesh

Patient Clinical status	vacA m-alleles		
	m1b	m2	m1b m2
PUD (n=112)	67	39	6
NUD (n=62)	25	25	12

5.5.4 Distribution of iceA alleles

PCR was used to test for *iceA1*, which is virulence associated in the West, and the completely unrelated *iceA2* gene, which can occupy the same chromosomal locus in strains lacking *iceA1*. The *iceA1* gene was in isolates from 58 of 112 patients with PU disease and in twenty-eight strains from patients with NUD. In contrast, only the *iceA2* gene was present in strains from 51 patients with PU and in 31 strains from patients with NUD. A mixture of *iceA1* and *iceA2* PCR products, indicating mixed infection, was found in six cultures out of a total of 174 (Table 27). A 94-bp deletion in the *iceA1* gene was found in two isolates, both from patients with PU.

Table 27:Distribution of iceA alleles in *H. pylori* strains from Bangladesh

Patient Clinical status	iceA-allels		
	iceA1	iceA2	iceA1, iceA2
PUD (n=112)	58	51	3
NUD (n=62)	28	31	3

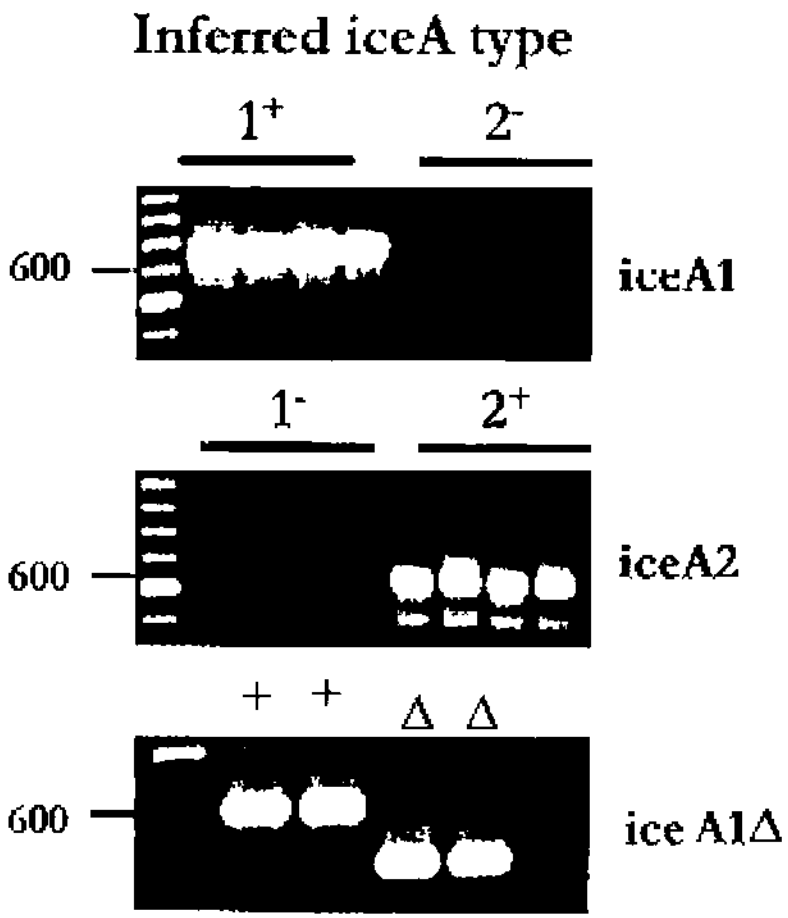


Fig15: Agarose gel electrophoresis showing PCR amplified productof *iceA1*,*iceA2* and 94bp deletion in *iceA*

6 Phylogenetic relationship between *cagA* 5' end of Bangladeshi isolates and those from other geographic regions

To assess the phylogenetic relationship between *cag* PAIs of Bangladeshi isolates and those from other regions, we sequenced a 219-bp fragment near the 5' end of the *cagA* gene. When the sequences of *cagA* gene from Bangladeshi *H. pylori* strains compared with the gene bank sequences of *cagA* gene from different countries, it was observed that the sequences of isolates from Bangladesh were closely related to each other and also to those from Western strains, but different from Chinese and Japanese strains (Fig.16).

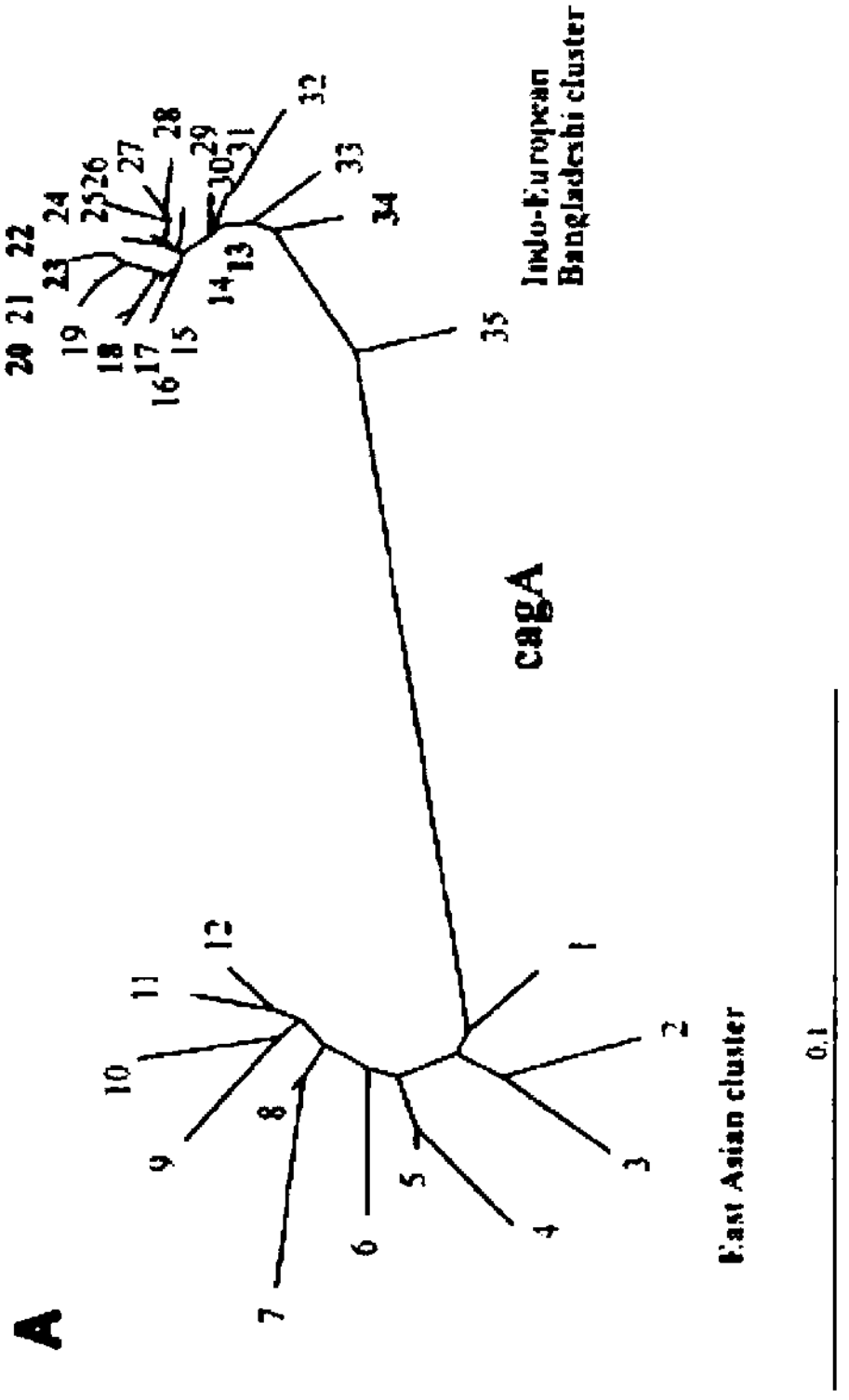


Fig. 16

6.1 Sequence analysis of mid-region alleles of *vacA*

DNAs of 0.7-kb PCR fragments amplified with *vacA* middle region-specific primers containing the *vacAm1b* region were sequenced for further analysis. The sequence was then compared with available *vacAm1a*, *-m1b*, and recently described *m1c* sequences in GenBank. Multiple sequence analysis showed all sequences from Bangladeshi isolates are closely related to each other and also closely related (97 to 98% identity) to a recently described *vacAm1c* allele from Calcutta, India (25). However, only 90 to 91% identity was obtained with both *vacAm1b* alleles from East Asia and *vacAm1a* of ethnic European strains. Phylogenetic analysis of the same sequence showed that Bangladeshi isolates are closely clustered to each other and are distantly related to East Asian and ethnic European clusters.

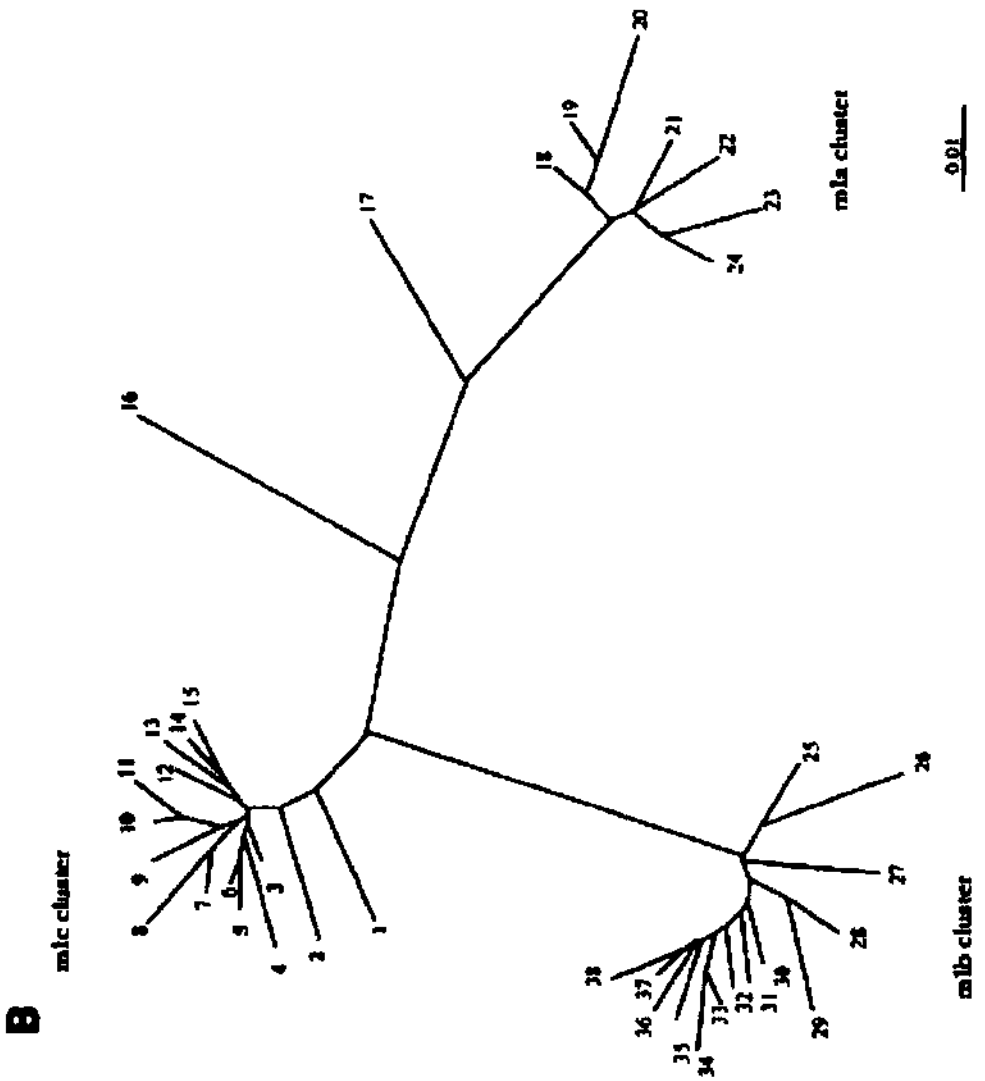


Fig. 17

Chapter VI
Discussion

The evidence that *H. pylori* cause gastritis in humans comes from both primary and secondary observations. The most important primary observations are the human volunteer studies, the animal models, and the treatment studies with antimicrobial agents. Supporting information comes from studies showing the specific association of *H. pylori* infection with type-B gastritis and with gastric (but not intestinal) epithelial cells; the specific ultra structural lesions, including adherence pedestals; the ubiquity and stability of the immune response; the response to bismuth treatment; and the association with epidemic gastritis and hypochlorhydria. It is important to note that all of Koch's postulates have been fulfilled, and despite nearly universal initial skepticism, no evidence exists against the hypothesis that *H. pylori* plays an etiologic role in type B gastritis. Therefore, it is reasonable to conclude that *H. pylori* are a pathogen in humans. *H. pylori* infection is chronic and common throughout the world, with a higher prevalence in developing countries than in developed countries. The prevalence of *H. pylori* infection increases with age in parallel with that of gastritis. Acquisition of *H. pylori* infection does not appear to have any seasonality, and infection is equally common among men and women. Without a significant animal or environmental reservoir for human strains of *H. pylori*, person-to-person contact appears to be the most likely mode of transmission. Exactly how the organism is transmitted from the stomach of one person to that of another remains unclear. Also unknown are the factors which determine who becomes ill after infection; why one person has gastritis alone while another person develops a duodenal ulcer; and how the traditional risk factors for ulcer disease, such as smoking, aspirin, and alcohol, interact with *H. pylori* infection. Finally, the long-term neoplastic consequence of infection is poorly understood. Further elucidation of the natural history of *H. pylori* and the consequences of *H. pylori* infection is the most important goal for future study.

Most of the information regarding *H. pylori* such as phenotypic characterization, methods for diagnosis, treatment regimen and outcome and genotypic characteristics was from the developed countries. As it is already mentioned that *H. pylori* is highly genetically diverse bacterium and all this factors and information varies from different parts of the world even in between developed countries. Information from developing countries, like Bangladesh, where there is a high prevalence of chronic infections is must to deal with this pathogen for better understanding and treatment with *H. pylori*

related illness. There is no single gold standard among the diagnostics tests for *H. pylori* infection but all of the tests have their pitfalls and limitations. We have studied the usefulness of the *H. pylori* stool antigen (HpSA) test for detection of *H. pylori* infection in young Bangladeshi children. Recommendations for diagnostic testing for *H. pylori* have been published by a number of different groups (110, 182). For the initial diagnosis, whole blood or serum serology is a cost-effective initial approach to testing for *H. pylori* in symptomatic or at-risk asymptomatic patients (i.e. history of peptic ulcer disease). However, it is appropriate to confirm a positive result using a different method in settings in which the pretest probability of infection is low (187). It has been suggested that the 13 C-UBT or the stool antigen test are good choices for patients who are not taking proton pump inhibitors, bismuth compounds or antibiotics, and that histology should be reserved for patients undergoing a diagnostic endoscopes or for those who require endoscopes. The accuracy of the stool test as a method for the primary diagnosis of *H. pylori* has been evaluated in several studies involving large groups of patients (237, 240).

In our study the EIA has been shown to be a useful non-invasive diagnostic test for *H. pylori* in children. Using the manufacturer's cut off level, the calculated sensitivity and specificity of the test was 78% and 99% respectively compared with ^{13}C UBT(urea breath test). The positive and negative predictive value and the accuracy of the test was 99%, 76% and 80% respectively. The assay has only recently been made available and this is one of the first published evaluations in children in Bangladesh as well as in developing countries. In previous studies in adults, sensitivity ranged from 89% (157) to 96% (145, 166) and specificity from 75% to 100% (40). To validate the EIA we compared it to the 13C UBT (Table 2).

The diagnosis of *H. pylori* infection can be based on endoscopy with biopsy and culture, but the ^{13}C UBT is also a sensitive indicator of the presence of the bacterium in the stomach and compares well with invasive testing (40). Colonization with *H. pylori* does not necessarily indicate disease; and tests with a high negative predictive value are useful to exclude infection whereas those with high positive predictive values indicate the need for further investigation. It is important to note that the negative predictive value depends on the cut off value chosen. We used the manufacturer's cut off which is based on adult studies. If the test is used to screen

patients, then choosing a cut off to maximize the negative predictive value may be appropriate. A test, which detects *H. pylori* antigen in feces may be of particular use in pediatric studies where non-invasive tests are preferred, and where obtaining blood samples, may proved to be difficult. The EIA also has several potential advantages over the UBT for population studies. Firstly, patients are not required to attend hospital as fecal specimens can be transported or posted to the laboratory. Secondly, no expensive instrumentation or expertise is required to perform a standard enzyme linked immunosorbent assay (ELISA) test. It costs only ~3 to 4 US$ for one test and the concordance of HpSA spectrophotometry and visual method is 100%, which makes the test even cheaper and easier. It is possible that delay between collection and analysis of stool might account for the lower sensitivity and specificity of the test. Our study did not include patients treated with antimicrobial or for other diseases. It is therefore not possible to comment on the effect that these factors may have on the EIA result. It is difficult to quantify the clinical significance of the cross-reaction with other *Helicobacter* species. However in a previous study *H. acinonyx* and *H. felis* showed a positive reaction when assayed by the EIA (221). *H. acinonyx* is genetically closely related to *H. pylori* (86) but there have been no reported cases of human infection. *H. felis* has been shown to cause human disease (81) and there was speculation that domestic cats might play a role in the transmission of infection (81), but a recent study suggests that owning pets is not a risk factor (23) for acquisition of *H. pylori* infection.

The definitive diagnosis of disease caused by *H. pylori* requires endoscopy. The UBT indicates the presence or absence of the organism in the stomach and is most appropriately used to confirm successful eradication of infection. With its high negative predictive value, ease of use, and non-invasive nature, the EIA is useful as a screening tool to exclude infection. The detection of its antigens in stool supports the hypothesis that *H. pylori* is excreted in the feces and might be the principal mode of transmission. However, stool antigen may be a product of digestion of the organism residing in the stomach. Therefore, other than few reports of culture from the stool (125, 232) there is little evidence that *H. pylori* survives passage through the gut. Nevertheless, detection of *H. pylori* antigens in the stool is likely to drive researchers with new determination to culture the organism from feces, and to try to characterize the form in which it exists in, or passes through the large bowel (248). Therefore, in

addition to its possible role as a diagnostic tool, the detection of stool antigens raises the possibility of genotyping the organism and thereby studying its transmission and epidemiology (248).

We conclude that stool HpSA test is reliable, rapid and easy way to exclude *H. pylori* infection in children in Bangladesh. The discrepancy between UBT and stool test for diagnosis of *H. pylori* infection requires further investigation.

The virulence factors and pathogenesis, which influence the clinical outcome of *H. pylori* infection, are still poorly understood. We have also documented the high prevalence of CagA and VacA seropositivity in asymptomatic Bangladeshi children with *H. pylori* infection. Using Western blot analysis, we observed a high seroprevalence of CagA and VacA indicating *H. pylori* infections in asymptomatic Bangladeshi children. Over 80% of the study children had or have had infections due to strains of *H. pylori* expressing CagA or VacA, or both as determined by Western blotting method. Among *H. pylori*-infected children, as diagnosed by positive urea breath test, the prevalence of antibody against CagA/VacA was even higher (94%). Presence of both cagA and vacA in the great majority of the study children with positive UBT indicates that gene coding for both the antigens are not only frequent but are also co-expressed in this community. The data from this study demonstrates that in this population, significant *H. pylori* infection begins in early life and most children had been exposed to *H. pylori* CagA and VacA antigens, and therefore developed specific IgG circulating antibodies to this virulence associated antigens. The findings of high seroprevalence of CagA and VacA in our study is consistent with reports from China (194, 222) Japan (154), and Thailand describing a high seroprevalence among adult patients with peptic ulcer, gastric cancer, and nonulcer dyspepsia. Similar results were also reported recently amongst dyspepsic Gambian adults and children, and Korean children undergoing upper endoscopy. It is important to note that, we detected similar prevalence of CagA and VacA antibodies amongst asymptomatic children in contrast to that reported amongst symptomatic children in those studies.

Phenotype I *H. pylori* strain, characterized by presence of both cagPAI and *vacA* gene, is believed to be more pathogenic compared to phenotype II strains that lack the

cag PAI and production of *VacA*, and are implicated in peptic ulcer diseases in Western adult population. In children, presence of abdominal pain was associated with infections caused by Cag-A positive *H. pylori* strains in those populations (207). Thus, our finding of very high prevalence of phenotype I strains in asymptomatic children is interesting. The differences between the Western and the Oriental strains of *H. pylori* add another dimension to the ongoing exploration of the relationship between the strain types of *H. pylori*, the clinical features and outcome of infections. The high prevalence of virulence markers in asymptomatic children most likely is associated with type-B gastritis, a condition that may remain asymptomatic in most infected individuals. A higher pepsinogen II, a well-known marker for gastritis, has also been demonstrated in children of Nandipara community (217).

Cag A-positive *H. pylori* infections have been found to increase the risk of gastric cancer by 3.2 folds, compared to infections due to *CagA*-negative *H. pylori* infections in western population (31, 196). Moreover, *CagA*-negative *H. pylori* infections were not significantly associated with cancer. We detected *Cag A*-positive strains in 82% of the children. If only *CagA* strains are indeed associated with cancers, then the finding of a very high prevalence of such strains in children of our study is alarming. However, the prevalence of duodenal ulcer and gastric cancer in this population has been reported to be very low- 10 and 0.5/1,000 hospitalized patients respectively (5, 175). We are unable to explain this disparity; however, the findings would indicate an enigma in developing countries and that genotype of the bacterium may not be the only risk factor for severe clinical illnesses as reported from the developed countries. Serum anti-*H. pylori* IgG determination is usually done for epidemiological studies because it is noninvasive and cost-effective. In our study, 95% of the children with a positive urea breath test were also positive for both of CagA and VacA by the Western Blot. Moreover, false-positive and false-negative results were seen with Western Blot at a very low rate, revealing an accuracy of 89% in diagnosing *H. pylori* infection. However, the specificity of Western blot was 68% indicating that in developing countries the infection, which is acquired in childhood, may undergo spontaneous clearance, mediated at least partly by specific immune mechanisms that also observed in our earlier study (217).

It is also possible, however, that the current wide spread nonspecific use of antibiotics may affect the potential contribution of naturally acquired immunity in the phenomenon of spontaneous *H. pylori* clearance as reported in developed countries. It has to be taken into account also that the UBT may as well produce inconsistent results after the intake of antibiotics, proton pump inhibitors, and gastric surgery and in children (41). Although urea breath test is non-invasive and usually indicates presence of active infection, high cost of the isotope 13/14 C urea may limit its application for epidemiological studies in the developing countries. Therefore, in settings with high prevalence of *H. pylori*, detection of CagA and VacA IgG could be a very useful tool for epidemiological studies. However the test results with Western Blot for *CagA* and *VacA* should be confirmed by UBT or by HpSA test. Western blot test in conjunction with urea breath test or stool antigen test may be desirable in for the purpose of diagnosis and identifying virulence factor in population where *Helicobacter pylori* infection is highly prevalent (217).

Impressive developments have occurred in the last decade regarding understanding of the genetics of *H. pylori* and some of the virulence factors involved in the pathogenic events such as colonization of *H. pylori* and *H. pylori*-mediated pathology have been described. In the development of an effective vaccine against *H. pylori*, it would be important to determine antigen that are prevalent in these target populations.

Further developments of effective vaccine(s) against *H. pylori* are expected to occur in the next couple of years. Recombinant *VacA*, native *CagA*, and orally administered formaldehyde-inactivated native *VacA* with either wild type LT or fully nontoxic mutant have already been found to completely prevent gastric colonization with the vacA+/cagA+ *H. pylori* strain in mice. Prophylactic and therapeutic protection has also been achieved with *CagA* following oral immunization with nontoxic LT mutant as an adjuvant in the same model. Consequent to these findings, several trials are envisaged in humans. The results of our study provide information on the prevalence of virulence factors of *H. pylori* among Bangladeshi population, which would be helpful in the development of effective vaccine(s) against *H. pylori* for prophylactic and therapeutic use.

In this multifaceted study we have tried to study the areas from phenotypes to genetics of *H. pylori*. We studied the demographic characteristics of individuals infected with *H. pylori* and tried to determine the relationship (if any) with disease outcome. We have enrolled a total of 278 patients (age range, 15 – 78 years) including 58% and 42% with PU and NUD, respectively. However, *H. pylori* prevalence by culture in this study was 63%, whereas previous reports from Bangladesh show that *H. pylori* prevalence ranges between 61% to 92% (218).

It has been thought that there were some relationships between ulcer development with smoking habits, alcohol consumptions, family history of peptic ulcer disease and food habits. To elucidate these relationships in this part of the world, we also collect in formations about this factor while enrolling the patients for this study.

No significant relationship between smoking habit and *H. pylori* infection was observed. Our results on the relation between smoking and *H. pylori* infection are similar to those from other studies. Despite differences in the populations studied and the methods used, most studies reported slightly increased odds of *H. pylori* infection among smokers, (19, 42, 78) but these odds were significant in only two studies (19, 78). These patterns are consistent with a possible weak increase in active *H. pylori* infection by smoking, which might be too subtle to be verified in most single epidemiological studies because of limited power.

The observed net association of smoking with active *H. pylori* infection may result from various mechanisms with partly antagonistic effects on the risk of infection. Potentially relevant effects of smoking include an increase in acid and pepsin secretion and changes in gastric motility, prostaglandin synthesis, gastric mucosal blood flow, and mucus secretion (70).

We did not find any significant relationship between alcohol consumption and *H. pylori* infection. Several studies have reported the relation between alcohol consumption and *H. pylori* infection (42, 59, 78, 93, 200, 213). Most of them did not find a significant association. A recent study from Finland showed a positive association between alcohol consumption and *H. pylori* infection among younger people (aged 18-35) and a negative association among older age groups (>46) (200).

Yet, interpretation and generalization of these findings are difficult because the study was conducted among military staff who had endoscopes for gastric complaints and who drank heavily.

A limitation of our study is the fact that information on alcohol consumption was ascertained without verification by biological markers. We believe that alcohol consumption, as in other epidemiological studies, has probably been underreported to some extent. Such underreporting could not have produced the observed patterns if alcohol consumption was unrelated to *H. pylori* infection (32).

We also did not find any significant relationship between family history as well as food habit and *H. pylori* infection, and the findings were similar to previous reports (219).

Although there are a number of diagnostic tests like invasive and non-invasive methods available for detecting whether a patient is infected with *H. pylori*, no single test is good enough at present to be the reference method or 'gold standard'. In this study, the patients were regarded as *H. pylori* positive if at least two test results were positive (169, 146, 230). Culture is the best method for *H. pylori* diagnosis, but due to unavailability of the culture system in our country stool antigen test, serum IgG is the best method for diagnosis of *H. pylori* infection.

Mammalian body do not produce urease enzyme, if samples contain urease that means patient possesses urease. *H. pylori* produces large amount of urease and presence of this enzyme in gastric mucosa usually indicates infection with this organism. Based on this rapid urease test (RUT) has been developed. In this study RUT was found to be positive in approximately 90% cases among the total patients. Of them the test showed positive results in 90.9% of the peptic ulcer patients 50% of non-ulcer dyspepsia patients and 20% cancer patients and all rapid urease test positive were found culture positive. Sometimes RUT shows negative results because biopsy tissues may contain lower number of bacteria, as about 10^5 colony forming units (CFU) needs to be present in a sample for a positive RUT test (168). In this study RUT was found to be highly correlating with culture. Also it was observed that RUT is the most sensitive and specific test for detection of *H. pylori* infection (9, 94).

Antimicrobial resistance in *H. pylori* is a growing problem as it is the most important factor in determining treatment outcome. The prevalence of antimicrobial resistance varies with geographical regions(55, 235). We also studied the antimicrobial susceptibility of *H. pylori* strains isolated in Bangladesh. Resistance to metronidazole was the most common type of resistance, with worldwide rates of 10 to 90% (55, 235). The high prevalence (77%) of metronidazole resistance in Bangladesh might be due to frequent use of metronidazole for other intestinal and gynecological problems. Previous use of metronidazole has been shown to be associated with *H. pylori* resistance to this antimicrobial agent (165). Two types of Mtzs *H. pylori* were isolated in the present study: type I, requiring only inactivation of rdxA to become resistant; and type II, requiring inactivation of both rdxA and frxA to become resistant. Only frxA inactivation did not have any role in metronidazole resistance, as only subsequent inactivation of frxA in rdxA-inactivated isolates reverted from the Mtzs phenotype to the Mtzr phenotype and increased the MIC for the Mtzr phenotype. This is in contrast to the findings of Kwon et al., who interpreted that the resistant phenotype can obtained by inactivation either of frxA or rdxA (141). Thus, resistance to metronidazole in *H. pylori* is mainly due to mutation in the rdxA gene and results from de novo mutation in the resident rdxA gene, rather than lateral transfer of a mutant rdxA gene.

The reported prevalence of primary resistance to clarithromycin ranges between 0 and 15% in most countries (55, 235). Around 10% of the isolates in the present study were clarithromycin resistant. In Bangladesh, clarithromycin was introduced in the late 1990s, and it has been widely used for eradication of *H. pylori*. Previous use of macrolides has been shown to be associated with *H. pylori* resistance to clarithromycin (165).

Amoxicillin resistance was not considered important until recently identified in the United States, Canada, and Italy (87, 181). Amoxicillin is one of the most commonly used antimicrobial agents in Bangladesh in recent years. Although 6.6% of the isolates were resistant, none was positive for β-lactamase. Amoxicillin resistance develops due to structural alterations in one of the penicillin-binding proteins (57) (60, 87) or changes in other proteins involved in cell wall synthesis (48, 156, 250),

and the resistant phenotype may be lost due to freezing or storage. All isolates tested in the present study were frozen at least once, and the low prevalence of the resistance phenotype may be due to loss during storage. Primary resistance to tetracycline ranges between 5 and 59% in Asian countries (141, 233, 251). Around 15% of the isolates in the present study were tetracycline resistant, which is in agreement with an earlier finding from this region.

Therefore, it is reasonable to conclude that in our geographical area, antibiotic resistance is an emerging problem for the treatment of *H. pylori*-infected patients. The present study also demonstrates the need for continuous monitoring of the antimicrobial susceptibility in *H. pylori* for determination of optimal treatment regimens.

An intriguing aspect of *H. pylori* is an extraordinary diversity among strains that is seen in DNA fingerprinting and in tests of gene content and chromosomal gene order (1, 3). Together with this are indications of differences at certain loci between strains from different parts of the world or human ethnic groups. There, we also studied the DNA-Level characterization of *H. pylori* strains from patients with overt disease and with benign infections in Bangladesh. *H. pylori* strains from Bangladesh were studied to gain new insight into the population genetic structure of the pathogen and to study the implication of genotype in disease. Although *H. pylori* infection occurs worldwide, there are significant differences in its prevalence both within and between countries (92, 170). In developing countries, including Bangladesh, the majority of children usually get infected during their first 1 to 2 years of life, and most others acquire *H. pylori* even before adulthood (135). An age-related prevalence study in Bangladesh showed that 61% of infants (1 to 3 months old) were positive for *H. pylori*, and the figure rose to 84% in 6- to 9-yearolds (155). Otherwise, the reported seroprevalence of *H. pylori* in the hospitalized Bangladeshi population was 77.4%(183). A high association of *H. pylori* with PU (duodenal ulcer, 77%; gastric ulcer, 75%) and gastritis (74%) was observed in a previous study (100). However, in the present study, *H. pylori* could be cultured from 56% of PU and 40% of patients with NUD reporting at Dhaka Medical College Hospital, Bangladesh. This may be due to a difference in methods for detection of *H. pylori* (other studies used serology, rapid-urease test or histology) and uneven distribution of the organism throughout the

gastric mucosa. Since the main focus of this study was to determine the genetic makeup in relation to virulence of Bangladeshi *H. pylori* strains and since culture of *H. pylori* from gastric biopsy specimens undoubtedly constitutes the most specific way to establish the presence of the bacteria and to study the genotype, it was not deemed necessary to employ all other methods for detection of *H. pylori* in the study.

H. pylori strains carrying the cag PAI and the potentially toxigenic s1 alleles of the vacuolating cytotoxin (*vacA*) gene were found to predominate in Bangladesh, but at the same time, some strains carrying the *vacA*s2 allele and lacking the cag PAI were recovered either as a sole pathogen infecting a patient or co resident with cag positive strains. The frequency at which strains carrying *vacAs2* allele and lacking cag PAI were found in Bangladesh was higher than that in East Asia, where essentially all strains studied to date have carried the cag PAI and *vacAs1* allele (118, 193). It is also noteworthy that strains lacking cag PAI were present in cases of both overt disease and more benign infections in Bangladesh, as opposed to the West, where such strains were associated only with benign infections (13). This, however, is similar to the situation in India, where cag positive *H. pylori* strains were harbored by both diseased patients and asymptomatic carriers (178). Mutational tests of strains from the West had shown that cag PAI genes are needed to strongly induce synthesis of proinflammatory cytokines in cell culture, and thus they are inferred to contribute to the strong and potentially damaging human host inflammatory responses that underlie overt disease (3). Hence, our results were unexpected. One explanation could be that infection by such strains in Bangladesh are often mixed with infections by strains with cag PAI, the latter being chiefly responsible for the observed severe pathology. A second explanation for the results invokes host or environmental factors or other bacterial genetic determinants that might be more important than cag^{+}vacAs1 status as determinants of disease in Bangladesh. A third possibility is suggested by indications that expression of cag PAI specified virulence functions may be highly controlled, with the severity of down-regulation varying among strains (50). Conceivably, this negative regulation might be more severe in most strains recovered from healthy individuals, effectively converting such nominally cag positive strains into phenocopies of strains lacking cag.

Similar to the Indian strains, iceA2 was found to be present in a large fraction of *H. pylori* strains isolated from patients with PU in Bangladesh, although iceA1 had been more strongly associated with disease in the U.S. and European populations studied to date (244). Expression of iceA1 is induced by gastric epithelial cell contact; its protein product is a homologue of a restriction endonuclease and is believed to cleave DNA in vitro (202)). IceA2 is completely unrelated to iceA1 in sequence, although the iceA1 and iceA2 genes occupy the same locus in different strains and antibodies against iceA2 are found in sera of some infected patients, implying that this protein is also produced during infection (77). Beyond these observations, however, little is known about the actual roles (if any) of iceA1 and iceA2 in human colonization and disease that would explain the basis of high iceA2 disease associations in Bangladesh.

DNA sequence phylogenetic analysis of the *vacA* middle region indicates that the strains from Bangladesh harbor a unique m1c allele that is common among isolates from the ethnic Bengali population in Calcutta (178). Studies on the distribution of neutral markers showed a significantly higher incidence of IS606, a 2-kb transposable element, in Bangladeshi strains compared to strains from China and Japan. IS606, which is found in one-third of strains from the United States and Europe but rarely in strains from East Asia (126), was present in 31.5% of the *H. pylori* strains from Bangladesh. This percentage is also higher than that observed for Indian strains, 17 to 18% of which normally carry IS606 (178).

A similarity between Bangladeshi and Indian strains was also reflected in the predominance of the type III motif at the cag PAI right junction. This motif was found in 85% of strains from Calcutta, whereas approximately 80% of Bangladeshi strains was positive for type III. Otherwise, the type III motif was uncommon elsewhere in the world.

The present study detected cases of mixed infection, i.e., the coexistence of genetically different *H. pylori* strains in a single infection in persons with overt disease as well as benign infections. This is important in the context that we humans differ in traits important to individual *H. pylori* strains, and that as a consequence, a given strain may not always be well adapted to the person who happens to ingest it. In

this framework, infections by strains of different and complementary genotypes should allow formation of recombinants that are better adapted than either parental

H. pylori continue to be one of the most common bacterial infections in humans. Functional genomics may fill many of the gaps in our understanding of the pathogenesis of *H. pylori* infection and accelerate the development of novel therapies, including *H. pylori*–specific antimicrobial agents. Although enormous progress has been made in studying the virulence factors of *H. pylori* and their variation, this information has not yet been used in clinical practice. Associations between bacterial characteristics and disease risks have not yet been defined sufficiently well to guide the clinician in treatment decisions. Prophylactic and therapeutic vaccination have been successful in animal models, but the translation to a human vaccine remains difficult, in part because the immunology of the stomach is still poorly understood (26, 171). All of these developments will probably be needed to prevent and treat this infection in areas of the world where there is a high prevalence of chronic infection. Strain to the given host (64, 127). Based on the presence or absence of cag PAI, seven subjects were found to have mixed infection; the number of mixed infections reported here may underestimate the true frequency in the Bangladeshi population. Because of the patchy distribution of the organism throughout the gastric mucosa, there exists considerable variation in the number of bacteria in biopsy specimens from different gastric sites. At the same time, *H. pylori* strains with different genotypes may predominate at different gastric sites, and any given biopsy specimen may contain only one strain or a subset of the mix of infecting strains that do not reflect the actual microenvironment of the host's stomach. Mixed *H. pylori* infection is also common in India.

It might be interesting to mention that when we looked at the house keeping genes *H. pylori* from Bangladesh are very similar isolates from west and east, but when virulence gene were analyzed they were very different, it indicated a host adaptation to infection and pathogenesis.

Thus, the overall genotypes of *H. pylori* from Bangladesh bear a very close resemblance to those of the strains from Calcutta, India. The high frequency of cag

positive vacAs1 strains, the sequence homology in vacAm1, the predominance of the type III motif, the occurrence of mixed infection, and the high frequency of IS606 were observed in strains of both the countries. This, however, is quite likely considering the close proximity of the two countries and the similar physiological environments, culinary practices, and lifestyles of the hosts.

The data of this study may provide us with a basis for further development of diagnosis and treatment against *H. pylori* infections in developing countries like Bangladesh.

References

1. **Achtman, M., T. Azuma, D. E. Berg, Y. Ito, G. Morelli, Z. J. Pan, S. Suerbaum, S. A. Thompson, A. van der Ende, and L. J. van Doorn.** 1999. Recombination and clonal groupings within *Helicobacter pylori* from different geographical regions. Mol Microbiol **32:**459-70.

2. **Ahmad, M. M., M. Rahman, A. K. Rumi, S. Islam, F. Huq, M. F. Chowdhury, F. Jinnah, M. G. Morshed, M. S. Hassan, A. K. Khan, and M. Hasan.** 1997. Prevalence of *Helicobacter pylori* in asymptomatic population--a pilot serological study in Bangladesh. J Epidemiol **7:**251-4.

3. **Akopyants, N. S., S. W. Clifton, D. Kersulyte, J. E. Crabtree, B. E. Youree, C. A. Reece, N. O. Bukanov, E. S. Drazek, B. A. Roe, and D. E. Berg.** 1998. Analyses of the cag pathogenicity island of *Helicobacter pylori*. Mol Microbiol **28:**37-53.

4. **Akopyanz, N., N. O. Bukanov, T. U. Westblom, S. Kresovich, and D. E. Berg.** 1992. DNA diversity among clinical isolates of *Helicobacter pylori* detected by PCR-based RAPD fingerprinting. Nucleic Acids Res **20:**5137-42.

5. **Alam, M. M.** 1995. Incidence of duodenal ulcer and its surgical management in a teaching hospital in Bangladesh. Trop Doct **25:**67-8.

6. **Aim, R. A., L. S. Ling, D. T. Moir, B. L. King, E. D. Brown, P. C. Doig, D. R. Smith, B. Noonan, B. C. Guild, B. L. deJonge, G. Carmel, P. J. Tummino, A. Caruso, M. Uria-Nickelsen, D. M. Mills, C. Ives, R. Gibson, D. Merberg, S. D. Mills, Q. Jiang, D. E. Taylor, G. F. Vovis, and T. J. Trust.** 1999. Genomic-sequence comparison of two unrelated isolates of the human gastric pathogen *Helicobacter pylori*. Nature **397:**176-80.

7. **Alm, R. A., and T. J. Trust.** 1999. Analysis of the genetic diversity of *Helicobacter pylori*: the tale of two genomes. J Mol Med **77:**834-46.

8. **Al-Qurashi, A. R., F. El-Morsy, and A. A. Al-Quorain.** 2001. Evolution of metronidazole and tetracycline susceptibility pattern in *Helicobacter pylori* at a hospital in Saudi Arabia. Int J Antimicrob Agents **17:**233-6.

9. **Andersen, L. P., S. Kiilerick, G. Pedersen, A. C. Thoreson, F. Jorgensen, J. Rath, N. E. Larsen, O. Borup, K. Krogfelt, J. Scheibel, and S. Rune.** 1998. An analysis of seven different methods to diagnose *Helicobacter pylori* infections. Scand J Gastroenterol **33:**24-30.

10. **Ando, T., T. M. Wassenaar, R. M. Peek, Jr., R. A. Aras, A. I. Tschumi, L. J. van Doorn, K. Kusugami, and M. J. Blaser.** 2002. A *Helicobacter pylori* restriction endonuclease-replacing gene, hrgA, is associated with gastric cancer in Asian strains. Cancer Res **62:**2385-9.

11. **Ando, T., Q. Xu, M. Torres, K. Kusugami, D. A. Israel, and M. J. Blaser.** 2000. Restriction-modification system differences in *Helicobacter pylori* are a barrier to interstrain plasmid transfer. Mol Microbiol **37:**1052-65.

12. **Ani, A. E., A. O. Malu, J. A. Onah, D. M. Queiroz, G. Kirschner, and G. A. Rocha.** 1999. Antimicrobial susceptibility test of *Helicobacter pylori* isolated from Jos, Nigeria. Trans R Soc Trop Med Hyg **93:**659-61.

13. **Atherton, J. C.** 1998. *H. pylori* virulence factors. Br Med Bull **54:**105-20.

14. **Atherton, J. C., P. Cao, R. M. Peek, Jr., M. K. Tummuru, M. J. Blaser, and T. L. Cover.** 1995. Mosaicism in vacuolating cytotoxin alleles of *Helicobacter pylori*. Association of specific vacA types with cytotoxin production and peptic ulceration. J Biol Chem **270:**17771-7.

15. **Atherton, J. C., and R. C. Spiller.** 1994. The urea breath test for *Helicobacter pylori*. Gut **35:**723-5.

16. **Bardhan, P. K.** 1997. Epidemiological features of *Helicobacter pylori* infection in developing countries. Clin Infect Dis **25:**973-8.

17. **Barr, M., M. Buckley, and C. O'Morain.** 2000. Review article: non-steroidal anti-inflammatory drugs and *Helicobacter pylori*. Aliment Pharmacol Ther **14 Suppl 3**:43-7.

18. **Bassily, S., R. W. Frenck, E. W. Mohareb, T. Wierzba, S. Savarino, E. Hall, A. Kotkat, A. Naficy, K. C. Hyams, and J. Clemens.** 1999. Seroprevalence of *Helicobacter pylori* among Egyptian newborns and their mothers: a preliminary report. Am J Trop Med Hyg **61**:37-40.

19. **Bateson, M. C.** 1993. Cigarette smoking and *Helicobacter pylori* infection. Postgrad Med J **69**:41-4.

20. **Beales, I. L.** 1998. *H. pylori*-associated hypochlorhydria. Gastroenterology **114**:618-21.

21. **Becker, S. I., R. D. Smalligan, J. D. Frame, H. Kleanthous, T. J. Tibbitts, T. P. Monath, and K. C. Hyams.** 1999. Risk of *Helicobacter pylori* infection among long-term residents in developing countries. Am J Trop Med Hyg **60**:267-70.

22. **Bereswill, S., S. Greiner, A. H. van Vliet, B. Waidner, F. Fassbinder, E. Schiltz, J. G. Kusters, and M. Kist**. 2000. Regulation of ferritin-mediated cytoplasmic iron storage by the ferric uptake regulator homolog (Fur) of *Helicobacter pylori*. J Bacteriol **182**:5948-53.

23. **Bereswill, S., U. Waidner, S. Odenbreit, F. Lichte, F. Fassbinder, G. Bode, and M. Kist.** 1998. Structural, functional and mutational analysis of the pfr gene encoding a ferritin from *Helicobacter pylori*. Microbiology **144 (Pt 9)**:2505-16.

24. **Berg, D. E., P. S. Hoffman, B. J. Appelmelk, and J. G. Kusters.** 1997. The *Helicobacter pylori* genome sequence: genetic factors for long life in the gastric mucosa. Trends Microbiol **5**:468-74.

25. **Bhan, M. K., R. Bahl, S. Sazawal, A. Sinha, R. Kumar, D. Mahalanabis, and J. D. Clemens.** 2002. Association between *Helicobacter pylori* infection and increased risk of typhoid fever. J Infect Dis **186:**1857-60.

26. **Blanchard, T. G., and S. J. Czinn.** 2000. Immunology of *Helicobacter pylori* and prospects for vaccine. Gastroenterol Clin North Am **29:**671-85.

27. **Blaser, M. J.** 1990. *Helicobacter pylori* and the pathogenesis of gastroduodenal inflammation. J Infect Dis **161:**626-33.

28. **Blaser, M. J.** 1998. *Helicobacters* are indigenous to the human stomach: duodenal ulceration is due to changes in gastric microecology in the modern era. Gut **43:**721-7.

29. **Blaser, M. J., and D. E. Berg.** 2001. *Helicobacter pylori* genetic diversity and risk of human disease. J Clin Invest **107:**767-73.

30. **Blaser, M. J., P. H. Chyou, and A. Nomura.** 1995. Age at establishment of *Helicobacter pylori* infection and gastric carcinoma, gastric ulcer, and duodenal ulcer risk. Cancer Res **55:**562-5.

31. **Blaser, M. J., G. I. Perez-Perez, H. Kleanthous, T. L. Cover, R. M. Peek, P. H. Chyou, G. N. Stemmermann, and A. Nomura.** 1995. Infection with *Helicobacter pylori* strains possessing cagA is associated with an increased risk of developing adenocarcinoma of the stomach. Cancer Res **55:**2111-5.

32. **Brenner, H., and D. Loomis.** 1994. Varied forms of bias due to nondifferential error in measuring exposure. Epidemiology **5:**510-7.

33. **Brown, L. M., T. L. Thomas, J. L. Ma, Y. S. Chang, W. C. You, W. D. Liu, L. Zhang, D. Pee, and M. H. Gail.** 2002. *Helicobacter pylori* infection in rural China: demographic, lifestyle and environmental factors. Int J Epidemiol **31:**638-45.

34. **Bukanov, N. O., and D. E. Berg.** 1994. Ordered cosmid library and high-resolution physical-genetic map of *Helicobacter pylori* strain NCTC11638. Mol Microbiol **11**:509-23.

35. **Burstein, M., E. Monge, R. Leon-Barua, R. Lozano, R. Berendson, R. H. Gilman, H. Legua, and C. Rodriguez.** 1991. Low peptic ulcer and high gastric cancer prevalence in a developing country with a high prevalence of infection by *Helicobacter pylori*. J Clin Gastroenterol **13**:154-6.

36. **Castro-Rodriguez, J. A., R. Leon-Barua, and M. Penny.** 1999. *Helicobacter pylori* is not a determinant factor of persistent diarrhoea or malnutrition in Peruvian children. Trans R Soc Trop Med Hyg **93**:537-9.

37. **Cederbrant, G., G. Kahlmeter, and A. Ljungh.** 1993. The E test for antimicrobial susceptibility testing of *Helicobacter pylori*. J Antimicrob Chemother **31**:65-71.

38. **Censini, S., C. Lange, Z. Xiang, J. E. Crabtree, P. Ghiara, M. Borodovsky, R. Rappuoli, and A. Covacci.** 1996. cag, a pathogenicity island of *Helicobacter pylori*, encodes type I-specific and disease-associated virulence factors. Proc Natl Acad Sci U S A **93**:14648-53.

39. **Chang, M. C., Y. T. Chang, C. T. Sun, M. S. Wu, H. P. Wang, and J. T. Lin.** 2002. Quantitative correlation of *Helicobacter pylori* stool antigen (HpSA) test with 13C-urea breath test (13C-UBT) by the updated Sydney grading system of gastritis. Hepatogastroenterology **49**:576-9.

40. **Chang, M. C., M. S. Wu, H. H. Wang, H. P. Wang, and J. T. Lin.** 1999. *Helicobacter pylori* stool antigen (HpSA) test--a simple, accurate and non-invasive test for detection of *Helicobacter pylori* infection. Hepatogastroenterology **46**:299-302.

41. **Chey, W. D.** 2000. Accurate diagnosis of *Helicobacter pylori*. 14C-urea breath test. Gastroenterol Clin North Am **29**:895-902.

42. **Chodos, J. E., B. M. Dworkin, F. Smith, K. Van Horn, L. Weiss, and W. S. Rosenthal.** 1988. *Campylobacter pylori* and gastroduodenal disease: a prospective endoscopic study and comparison of diagnostic tests. Am J Gastroenterol **83:**1226-30.

43. **Clancy, S. M., and W. J. Hoyer.** 1993. Skill and hemispheric specialization in detecting featural differences in visual images. Brain Cogn **21:**192-202.

44. **Clemens, J., M. J. Albert, M. Rao, S. Huda, F. Qadri, F. P. Van Loon, B. Pradhan, A. Naficy, and A. Banik.** 1996. Sociodemographic, hygienic and nutritional correlates of *Helicobacter pylori* infection of young Bangladeshi children. Pediatr Infect Dis J **15:**1113-8.

45. **Clemens, J., M. J. Albert, M. Rao, F. Qadri, S. Huda, B. Kay, F. P. van Loon, D. Sack, B. A. Pradhan, and R. B. Sack.** 1995. Impact of infection by *Helicobacter pylori* on the risk and severity of endemic cholera. J Infect Dis **171:**1653-6.

46. **Copass, M., G. Grandi, and R. Rappuoli.** 1997. Introduction of unmarked mutations in the *Helicobacter pylori* vacA gene with a sucrose sensitivity marker. Infect Immun **65:**1949-52.

47. **Corthesy-Theulaz, I.** 2000. Vaccination against *Helicobacter pylori*. Recent Results Cancer Res **156:**55-9.

48. **Costa, C. S., and D. N. Anton.** 1993. Round-cell mutants of *Salmonella typhimurium* produced by transposition mutagenesis: lethality of rodA and mre mutations. Mol Gen Genet **236:**387-94.

49. **Covacci, A., J. L. Telford, G. Del Giudice, J. Parsonnet, and R. Rappuoli.** 1999. *Helicobacter pylori* virulence and genetic geography. Science **284:**1328-33.

50. **Crabtree, J. E., D. Kersulyte, S. D. Li, I. J. Lindley, and D. E. Berg.** 1999. Modulation of *Helicobacter pylori* induced interleukin-8 synthesis in gastric

epithelial cells mediated by cag PAI encoded VirD4 homologue. J Clin Pathol **52:**653-7.

51. **Crabtree, J. E., T. M. Shallcross, R. V. Heatley, and J. I. Wyatt.** 1991. Evaluation of a commercial ELISA for serodiagnosis of *Helicobacter pylori* infection. J Clin Pathol **44:**326-8.

52. **Crabtree, J. E., Z. Xiang, I. J. Lindley, D. S. Tompkins, R. Rappuoli, and A. Covacci.** 1995. Induction of interleukin-8 secretion from gastric epithelial cells by a cagA negative isogenic mutant of *Helicobacter pylori*. J Clin Pathol **48:**967-9.

53. **Cruickshank, J. K., P. W. Barritt, F. McBesag, N. Waterhouse, and L. H. Goldman.** 1975. Student views on continuous assessment at Birmingham University Medical School. Br Med J **4:**265-7.

54. **De Pina, K., V. Desjardin, M. A. Mandrand-Berthelot, G. Giordano, and L. F. Wu.** 1999. Isolation and characterization of the nikR gene encoding a nickel-responsive regulator in Escherichia coli. J Bacteriol **181:**670-4.

55. **Debets-Ossenkopp, Y. J., A. J. Herscheid, R. G. Pot, E. J. Kuipers, J. G. Kusters, and C. M. Vandenbroucke-Grauls.** 1999. Prevalence of *Helicobacter pylori* resistance to metronidazole, clarithromycin, amoxycillin, tetracycline and trovafloxacin in The Netherlands. J Antimicrob Chemother **43:**511-5.

56. **DeCross, A. J., and D. M. Sahasrabudhe.** 1992. Paraneoplastic Raynaud's phenomenon. Am J Med **92:**571-2.

57. **DeLoney, C. R., and N. L. Schiller.** 2000. Characterization of an In vitro-selected amoxicillin-resistant strain of *Helicobacter pylori*. Antimicrob Agents Chemother **44:**3368-73.

58. **Dominici, P., S. Bellentani, A. R. Di Biase, G. Saccoccio, A. Le Rose, F. Masutti, L. Viola, F. Balli, C. Tiribelli, R. Grilli, M. Fusillo, and E. Grossi.**

1999. Familial clustering of *Helicobacter pylori* infection: population based study. Bmj **319:**537-40.

59. **Dooley, C. P., H. Cohen, P. L. Fitzgibbons, M. Bauer, M. D. Appleman, G. I. Perez-Perez, and M. J. Blaser.** 1989. Prevalence of *Helicobacter pylori* infection and histologic gastritis in asymptomatic persons. N Engl J Med **321:**1562-6.

60. **Dore, M. P., D. Y. Graham, and A. R. Sepulveda.** 1999. Different penicillin-binding protein profiles in amoxicillin-resistant *Helicobacter pylori*. Helicobacter **4:**154-61.

61. **Dore, M. P., H. M. Malaty, D. Y. Graham, G. Fanciulli, G. Delitala, and G. Realdi.** 2002. Risk Factors Associated with *Helicobacter pylori* Infection among Children in a Defined Geographic Area. Clin Infect Dis **35:**240-5.

62. **Douce, G., M. Fontana, M. Pizza, R. Rappuoli, and G. Dougan.** 1997. Intranasal immunogenicity and adjuvanticity of site-directed mutant derivatives of cholera toxin. Infect Immun **65:**2821-8.

63. **Dowsett, S. A., L. Archila, V. A. Segreto, C. R. Gonzalez, A. Silva, K. A. Vastola, R. D. Bartizek, and M. J. Kowolik.** 1999. *Helicobacter pylori* infection in indigenous families of Central America: serostatus and oral and fingernail carriage. J Clin Microbiol **37:**2456-60.

64. **Dubois, A., D. E. Berg, E. T. Incecik, N. Fiala, L. M. Heman-Ackah, J. Del Valle, M. Yang, H. P. Wirth, G. I. Perez-Perez, and M. J. Blaser.** 1999. Host specificity of *Helicobacter pylori* strains and host responses in experimentally challenged nonhuman primates. Gastroenterology **116:**90-6.

65. **Dummer, J. S., G. I. Perez-Perez, M. K. Breinig, A. Lee, S. N. Wolff, R. Kormos, B. P. Griffith, and M. J. Blaser.** 1995. Seroepidemiology of *Helicobacter pylori* infection in heart transplant recipients. Clin Infect Dis **21:**1303-5.

66. **Dunn, B. E., H. Cohen, and M. J. Blaser.** 1997. *Helicobacter pylori.* Clin Microbiol Rev **10:**720-41.

67. **Eaton, K. A., C. E. Catrenich, K. M. Makin, and S. Krakowka.** 1995. Virulence of coccoid and bacillary forms of *Helicobacter pylori* in gnotobiotic piglets. J Infect Dis **171:**459-62.

68. **Eaton, K. A., and S. Krakowka.** 1995. Avirulent, urease-deficient *Helicobacter pylori* colonizes gastric epithelial explants ex vivo. Scand J Gastroenterol **30:**434-7.

69. **Eaton, K. A., and S. Krakowka.** 1994. Effect of gastric pH on urease-dependent colonization of gnotobiotic piglets by *Helicobacter pylori.* Infect Immun **62:**3604-7.

70. **Endoh, K., and F. W. Leung.** 1994. Effects of smoking and nicotine on the gastric mucosa: a review of clinical and experimental evidence. Gastroenterology **107:**864-78.

71. **Eyitayo, C. A., A. O. Coker, R. Adegbola, T. Johnson, D. K. Olukoya, and T. Odugbemi.** 1992. Antibiotic susceptibility patterns and plasmid profile of Helicobacter isolates from Nigeria. West Afr J Med **11:**190-4.

72. **Falk, G. W.** 2001. GERD and *H. pylori*: is there a link? Semin Gastrointest Dis **12:**16-25.

73. **Falk, G. W.** 2001. The possible role of *Helicobacter pylori* in GERD. Semin Gastrointest Dis **12:**186-95.

74. **Fan, X. G., A. Chua, T. G. Li, and Q. S. Zeng.** 1998. Survival of *Helicobacter pylori* in milk and tap water. J Gastroenterol Hepatol **13:**1096-8.

75. **Fennerty, M. B.** 2001. Is nonulcer dyspepsia related to *Helicobacter pylori* infection? Semin Gastrointest Dis **12:**180-5.

76. **Ferrero, R. L., V. Cussac, P. Courcoux, and A. Labigne.** 1992. Construction of isogenic urease-negative mutants of *Helicobacter pylori* by allelic exchange. J Bacteriol **174:**4212-7.

77. **Figueiredo, C., W. G. Quint, R. Sanna, E. Sablon, J. P. Donahue, Q. Xu, G. G. Miller, R. M. Peek, Jr., M. J. Blaser, and L. J. van Doorn.** 2000. Genetic organization and heterogeneity of the iceA locus of *Helicobacter pylori*. Gene **246:**59-68.

78. **Fontham, E. T., B. Ruiz, A. Perez, F. Hunter, and P. Correa.** 1995. Determinants of *Helicobacter pylori* infection and chronic gastritis. Am J Gastroenterol **90:**1094-101.

79. **Forman, D., D. G. Newell, F. Fullerton, J. W. Yarnell, A. R. Stacey, N. Wald, and F. Sitas.** 1991. Association between infection with *Helicobacter pylori* and risk of gastric cancer: evidence from a prospective investigation. Bmj **302:**1302-5.

80. **Forsyth, M. H., and T. L. Cover.** 2000. Intercellular communication in *Helicobacter pylori*: luxS is essential for the production of an extracellular signaling molecule. Infect Immun **68:**3193-9.

81. **Fox, J. G.** 1995. Non-human reservoirs of *Helicobacter pylori*. Aliment Pharmacol Ther 9 **Suppl 2:**93-103.

82. **Ganga-Zandzou, P. S., L. Michaud, P. Vincent, M. O. Husson, N. Wizla-Derambure, E. M. Delassalle, D. Turck, and F. Gottrand.** 1999. Natural outcome of *Helicobacter pylori* infection in asymptomatic children: a two-year follow-up study. Pediatrics **104:**216-21.

83. **Garner, J. A., and T. L. Cover.** 1995. Analysis of genetic diversity in cytotoxin-producing and non-cytotoxin-producing *Helicobacter pylori* strains. J Infect Dis **172:**290-3.

84. **Garza-Gonzalez, E., G. I. Perez-Perez, O. Alanis-Aguilar, R. Tijerina-Menchaca, H. J. Maldonado-Garza, and F. J. Bosques-Padilla.** 2002. Antibiotic susceptibility patterns of *Helicobacter pylori* strains isolated from northeastern Mexico. J Chemother **14:**342-5.

85. **Gerhard, M., N. Lehn, N. Neumayer, T. Boren, R. Rad, W. Schepp, S. Miehlke, M. Classen, and C. Prinz.** 1999. Clinical relevance of the *Helicobacter pylori* gene for blood-group antigen-binding adhesin. Proc Natl Acad Sci U S A **96:**12778-83.

86. **Germani, Y., C. Dauga, P. Duval, M. Huerre, M. Levy, G. Pialoux, P. Sansonetti, and P. A. Grimont.** 1997. Strategy for the detection of Helicobacter species by amplification of 16S rRNA genes and identification of H. felis in a human gastric biopsy. Res Microbiol **148:**315-26.

87. **Gerrits, M. M., D. Schuijffel, A. A. van Zwet, E. J. Kuipers, C. M. Vandenbroucke-Grauls, and J. G. Kusters.** 2002. Alterations in penicillin-binding protein 1A confer resistance to beta-lactam antibiotics in *Helicobacter pylori.* Antimicrob Agents Chemother **46:**2229-33.

88. **Glassman, M. S., S. Dallal, S. H. Berezin, H. E. Bostwick, L. J. Newman, G. I. Perez-Perez, and M. J. Blaser.** 1990. *Helicobacter pylori*-related gastroduodenal disease in children. Diagnostic utility of enzyme-linked immunosorbent assay. Dig Dis Sci **35:**993-7.

89. **Glynn, M. K., C. R. Friedman, B. D. Gold, B. Khanna, L. Hutwagner, N. Iihoshi, C. Revollo, and R. Quick.** 2002. Seroincidence of *Helicobacter pylori* infection in a cohort of rural Bolivian children: acquisition and analysis of possible risk factors. Clin Infect Dis **35:**1059-65.

90. **Go, M. F.** 2002. Review article: natural history and epidemiology of *Helicobacter pylori* infection. Aliment Pharmacol Ther **16 Suppl 1:**3-15.

91. **Goh, K. L., and N. Parasakthi.** 2001. The racial cohort phenomenon: seroepidemiology of *Helicobacter pylori* infection in a multiracial South-East Asian country. Eur J Gastroenterol Hepatol **13:**177-83.

92. **Goodman, K. J., and P. Correa.** 1995. The transmission of *Helicobacter pylori*. A critical review of the evidence. Int J Epidemiol **24:**875-87.

93. **Graham, D. Y., H. M. Malaty, D. G. Evans, D. J. Evans, Jr., P. D. Klein, and E. Adam.** 1991. Epidemiology of *Helicobacter pylori* in an asymptomatic population in the United States. Effect of age, race, and socioeconomic status. Gastroenterology **100:**1495-501.

94. **Grove, D. I., G. Koutsouridis, and A. G. Cummins.** 1998. Comparison of culture, histopathology and urease testing for the diagnosis of *Helicobacter pylori* gastritis and susceptibility to amoxycillin, clarithromycin, metronidazole and tetracycline. Pathology **30:**183-7.

95. **Grubel, P., J. S. Hoffman, F. K. Chong, N. A. Burstein, C. Mepani, and D. R. Cave.** 1997. Vector potential of houseflies (Musca domestica) for *Helicobacter pylori*. J Clin Microbiol **35:**1300-3.

96. **Grubel, P., L. Huang, N. Masubuchi, F. J. Stutzenberger, and D. R. Cave.** 1998. Detection of *Helicobacter pylori* DNA in houseflies (Musca domestica) on three continents. Lancet **352:**788-9.

97. **Haas, R., T. F. Meyer, and J. P. van Putten.** 1993. Aflagellated mutants of *Helicobacter pylori* generated by genetic transformation of naturally competent strains using transposon shuttle mutagenesis. Mol Microbiol **8:**753-60.

98. **Hachem, C. Y., J. E. Clarridge, R. Reddy, R. Flamm, D. G. Evans, S. K. Tanaka, and D. Y. Graham.** 1996. Antimicrobial susceptibility testing of *Helicobacter pylori*. Comparison of E-test, broth microdilution, and disk

diffusion for ampicillin, clarithromycin, and metronidazole. Diagn Microbiol Infect Dis 24:37-41.

99. **Hallisey, M. J., and V. J. Caride.** 1990. Single perfusion defect secondary to intrathoracic kidney. Clin Nucl Med **15:**523-**4**.

100. **Haque, M., K. Masihur Rahman, A. Azad Khan, M. Hasan, M. R. Amin Miah, T. Rahman, A. K. Khurshed Alam, and F. Qadri.** 1995. Isolation and characterization of *Helicobacter pylori* strains from peptic ulcer patients in Dhaka, Bangladesh. Indian J Gastroenterol **14:**128-30.

101. **Harford, W. V., C. Barnett, E. Lee, G. Perez-Perez, M. J. Blaser, and W. L. Peterson.** 2000. Acute gastritis with hypochlorhydria: report of 35 cases with long term follow up. Gut **47:**467-72.

102. **Heuermann, D., and R. Haas.** 1995. Genetic organization of a small cryptic plasmid of *Helicobacter pylori*. Gene **165:**17-24.

103. **Heuermann, D., and R. Haas.** 1998. A stable shuttle vector system for efficient genetic complementation of *Helicobacter pylori* strains by transformation and conjugation. Mol Gen Genet **257:**519-28.

104. **Hildebrand, P., P. Bardhan, L. Rossi, S. Parvin, A. Rahman, M. S. Arefin, M. Hasan, M. M. Ahmad, K. Glatz-Krieger, L. Terracciano, P. Bauerfeind, C. Beglinger, N. Gyr, and A. K. Khan.** 2001. Recrudescence and reinfection with *Helicobacter pylori* after eradication therapy in Bangladeshi adults. Gastroenterology **121:**792-8.

105. **Hirayama, F., S. Takagi, Y. Yokoyama, E. Iwao, and Y. Ikeda.** 1996. Establishment of gastric *Helicobacter pylori* infection in Mongolian gerbils. J Gastroenterol **31 Suppl 9:**24-8.

106. **Hook-Nikanne, J.** 1991. Effect of alcohol consumption on the risk of *Helicobacter pylori* infection. Digestion **50:**92-8.

107. **Hook-Nikanne, J., D. E. Berg, R. M. Peek, Jr., D. Kersulyte, M. K. Tummuru, and M. J. Blaser.** 1998. DNA sequence conservation and diversity in transposable element IS605 of *Helicobacter pylori*. Helicobacter **3:**79-85.

108. **Hopkins, R. J., and J. G. Morris, Jr.** 1994. *Helicobacter pylori*: the missing link in perspective. Am J Med **97:**265-77.

109. **Hopkins, R. J., P. A. Vial, C. Ferreccio, J. Ovalle, P. Prado, V. Sotomayor, R. G. Russell, S. S. Wasserman, and J. G. Morris, Jr.** 1993. Seroprevalence of *Helicobacter pylori* in Chile: vegetables may serve as one route of transmission. J Infect Dis **168:**222-6.

110. **Howden, C. W., and R. H. Hunt.** 1998. Guidelines for the management of *Helicobacter pylori* infection. Ad Hoc Committee on Practice Parameters of the American College of Gastroenterology. Am J Gastroenterol **93:**2330-8.

111. **Hu, P. J., Y. Y. Li, H. L. Lin, S. M. Zhou, G. Du, M. H. Chen, H. M. Mitchell, and S. L. Hazell.** 1995. Gastric atrophy and regional variation in upper gastrointestinal disease. Am J Gastroenterol **90:**1102-6.

112. **Hu, P. J., Y. Y. Li, M. H. Zhou, M. H. Chen, G. G. Du, B. J. Huang, H. M. Mitchell, and S. L. Hazell.** 1995. *Helicobacter pylori* associated with a high prevalence of duodenal ulcer disease and a low prevalence of gastric cancer in a developing nation. Gut **36:**198-202.

113. **Huesca, M., A. Goodwin, A. Bhagwansingh, P. Hoffman, and C. A. Lingwood.** 1998. Characterization of an acidic-pH-inducible stress protein (hsp70), a putative sulfatide binding adhesin, from *Helicobacter pylori*. Infect Immun **66:**4061-7.

114. **Huynen, M., T. Dandekar, and P. Bork.** 1998. Differential genome analysis applied to the species-specific features of *Helicobacter pylori*. FEBS Lett **426:**1-5.

115. **Hyams, K. C., D. N. Taylor, G. C. Gray, J. B. Knowles, R. Hawkins, and J. D. Malone.** 1995. The risk of *Helicobacter pylori* infection among U.S. military personnel deployed outside the United States. Am J Trop Med Hyg **52:**109-12.

116. **Iiver, D., A. Arnqvist, J. Ogren, I. M. Frick, D. Kersulyte, E. T. Incecik, D. E. Berg, A. Covacci, L. Engstrand, and T. Boren.** 1998. *Helicobacter pylori* adhesin binding fucosylated histo-blood group antigens revealed by retagging. Science **279:**373-7.

117. **Isenbarger, D. W., L. Bodhidatta, C. W. Hoge, W. Nirdnoy, C. Pitarangsi, U. Umpawasiri, and P. Echeverria.** 1998. Prospective study of the incidence of diarrheal disease and *Helicobacter pylori* infection among children in an orphanage in Thailand. Am J Trop Med Hyg **59:**796-800.

118. **Ito, Y., T. Azuma, S. Ito, H. Suto, H. Miyaji, Y. Yamazaki, Y. Kohli, and M. Kuriyama.** 1998. Full-length sequence analysis of the vacA gene from cytotoxic and noncytotoxic *Helicobacter pylori.* J Infect Dis **178:**1391-8.

119. **Jeong, J. Y., A. K. Mukhopadhyay, D. Dailidiene, Y. Wang, B. Velapatino, R. H. Gilman, A. J. Parkinson, G. B. Nair, B. C. Wong, S. K. Lam, R. Mistry, I. Segal, Y. Yuan, H. Gao, T. Alarcon, M. L. Brea, Y. Ito, D. Kersulyte, H. K. Lee, Y. Gong, A. Goodwin, P. S. Hoffman, and D. E. Berg.** 2000. Sequential inactivation of rdxA (HP0954) and frxA (HP0642) nitroreductase genes causes moderate and high-level metronidazole resistance in *Helicobacter pylori.* J Bacteriol **182:**5082-90.

120. **Joyce, E. A., B. L. Bassler, and A. Wright.** 2000. Evidence for a signaling system in *Helicobacter pylori*: detection of a luxS-encoded autoinducer. J Bacteriol **182:**3638-43.

121. **Kansau, I., J. Raymond, E. Bingen, P. Courcoux, N. Kalach, M. Bergeret, N. Braimi, C. Dupont, and A. Labigne.** 1996. Genotyping of *Helicobacter*

pylori isolates by sequencing of PCR products and comparison with the RAPD technique. Res Microbiol **147:**661-9.

122. **Kauser, F., A. A. Khan, M. A. Hussain, I. M. Carroll, N. Ahmad, S. Tiwari, Y. Shouche, B. Das, M. Alam, S. M. Ali, C. M. Habibullah, R. Sierra, F. Megraud, L. A. Sechi, and N. Ahmed.** 2004. The cag pathogenicity island of *Helicobacter pylori* is disrupted in the majority of patient isolates from different human populations. J Clin Microbiol **42:**5302-8.

123. **Kawakami, E., S. K. Ogata, A. C. Portorreal, A. M. Magni, M. L. Pardo, and F. R. Patricio.** 2001. Triple therapy with clarithromycin, amoxicillin and omeprazole for *Helicobacter pylori* eradication in children and adolescents. Arq Gastroenterol **38:**203-6.

124. **Keates, S., Y. S. Hitti, M. Upton, and C. P. Kelly.** 1997. *Helicobacter pylori* infection activates NF-kappa B in gastric epithelial cells. Gastroenterology **113:**1099-109.

125. **Kelly, S. M., M. C. Pitcher, S. M. Farmery, and G. R. Gibson.** 1994. Isolation of *Helicobacter pylori* from feces of patients with dyspepsia in the United Kingdom. Gastroenterology **107:**1671-4.

126. **Kersulyte, D., N. S. Akopyants, S. W. Clifton, B. A. Roe, and D. E. Berg.** 1998. Novel sequence organization and insertion specificity of IS605 and IS606: chimaeric transposable elements of *Helicobacter pylori.* Gene **223:**175-86.

127. **Kersulyte, D., H. Chalkauskas, and D. E. Berg.** 1999. Emergence of recombinant strains of *Helicobacter pylori* during human infection. Mol Microbiol **31:**31-43.

128. **Kersulyte, D., A. K. Mukhopadhyay, M. Shirai, T. Nakazawa, and D. E. Berg.** 2000. Functional organization and insertion specificity of IS607, a chimeric element of *Helicobacter pylori.* J Bacteriol **182:**5300-8.

129. **Kersulyte, D., A. K. Mukhopadhyay, B. Velapatino, W. Su, Z. Pan, C. Garcia, V. Hernandez, Y. Valdez, R. S. Mistry, R. H. Gilman, Y. Yuan, H. Gao, T. Alarcon, M. Lopez-Brea, G. Balakrish Nair, A. Chowdhury, S. Datta, M. Shirai, T. Nakazawa, R. Ally, I. Segal, B. C. Wong, S. K. Lam, F. O. Olfat, T. Boren, L. Engstrand, O. Torres, R. Schneider, J. E. Thomas, S. Czinn, and D. E. Berg.** 2000. Differences in genotypes of *Helicobacter pylori* from different human populations. J Bacteriol **182:**3210-8.

130. **Kersulyte, D., B. Velapatino, G. Dailide, A. K. Mukhopadhyay, Y. Ito, L. Cahuayme, A. J. Parkinson, R. H. Gilman, and D. E. Berg.** 2002. Transposable element ISHp608 of *Helicobacter pylori*: nonrandom geographic distribution, functional organization, and insertion specificity. J Bacteriol **184:**992-1002.

131. **Kidd, M., and I. M. Modlin.** 1998. A century of *Helicobacter pylori*: paradigms lost-paradigms regained. Digestion **59:1-15.**

132. **Kim, J. S., J. H. Chang, S. I. Chung, and J. S. Yum.** 1999. Molecular cloning and characterization of the *Helicobacter pylori* fliD gene, an essential factor in flagellar structure and motility. J Bacteriol **181:**6969-76.

133. **Kitagawa, M., M. Natori, M. Katoh, K. Sugimoto, H. Omi, Y. Akiyama, and H. Sago.** 2001. Maternal transmission of *Helicobacter pylori* in the perinatal period. J Obstet Gynaecol Res **27:**225-30.

134. **Kleanthous, H., C. L. Clayton, and S. Tabaqchali.** 1991. Characterization of a plasmid from *Helicobacter pylori* encoding a replication protein common to plasmids in gram-positive bacteria. Mol Microbiol **5:**2377-89.

135. **Klein, P. D., R. H. Gilman, R. Leon-Barua, F. Diaz, E. O. Smith, and D. Y. Graham.** 1994. The epidemiology of *Helicobacter pylori* in Peruvian children between 6 and 30 months of age. Am J Gastroenterol **89:**2196-200.

136. **Klein, P. D., D. Y. Graham, A. Gaillour, A. R. Opekun, and E. O. Smith.** 1991. Water source as risk factor for *Helicobacter pylori* infection in Peruvian children. Gastrointestinal Physiology Working Group. Lancet **337:**1503-6.

137. **Kobayashi, T., K. Honke, I. Tsunematsu, H. Kagaya, S. Nishikawa, K. Hokari, M. Kato, H. Takeda, T. Sugiyama, A. Higuchi, and M. Asaka.** 1999. Detection of cerebroside sulfotransferase mRNA in human gastric mucosa and adenocarcinoma. Cancer Lett **138:**45-51.

138. **Kosunen, T. U., K. Seppala, S. Sarna, and P. Sipponen.** 1992. Diagnostic value of decreasing IgG, IgA, and IgM antibody titres after eradication of *Helicobacter pylori*. Lancet **339:**893-5.

139. **Krakowka, S., K. A. Eaton, D. M. Rings, and D. R. Morgan.** 1991. Gastritis induced by *Helicobacter pylori* in gnotobiotic piglets. Rev Infect Dis **13 Suppl 8:**S681-5.

140. **Kuipers, E. J., and G. A. Meijer.** 2000. *Helicobacter pylori* gastritis in Africa. Eur J Gastroenterol Hepatol **12:**601-3.

141. **Kwon, D. H., F. A. El-Zaatari, M. Kato, M. S. Osato, R. Reddy, Y. Yamaoka, and D. Y. Graham.** 2000. Analysis of rdxA and involvement of additional genes encoding NAD(P)H flavin oxidoreductase (FrxA) and ferredoxin-like protein (FdxB) in metronidazole resistance of *Helicobacter pylori*. Antimicrob Agents Chemother 44:2133-42.

142. **Lambert, J. R., S. K. Lin, and J. Aranda-Michel.** 1995. *Helicobacter pylori*. Scand J Gastroenterol Suppl **208:**33-46.

143. **Lanciers, S., B. Hauser, Y. Vandenplas, and U. Blecker.** 1996. The prevalence of *Helicobacter pylori* positivity in asymptomatic children of different ethnic backgrounds living in the same country. Ethn Health **1:**169-73.

144. **Lee, C. K., K. Soike, J. Hill, K. Georgakopoulos, T. Tibbitts, J. Ingrassia, H. Gray, J. Boden, H. Kleanthous, P. Giannasca, T. Ermak, R. Weltzin, J. Blanchard, and T. P. Monath.** 1999. Immunization with recombinant *Helicobacter pylori* urease decreases colonization levels following experimental infection of rhesus monkeys. Vaccine **17:**1493-505.

145. **Lehmann, F., J. Drewe, L. Terracciano, R. Stuber, R. Frei, and C. Beglinger.** 1999. Comparison of stool immunoassay with standard methods for detecting *Helicobacter pylori* infection. Bmj **319:**1409.

146. **Lerang, F., B. Moum, P. Mowinckel, J. B. Haug, E. Ragnhildstveit, T. Berge, and A. Bjorneklett.** 1998. Accuracy of seven different tests for the diagnosis of *Helicobacter pylori* infection and the impact of H2-receptor antagonists on test results. Scand J Gastroenterol **33:**364-9.

147. **Lindkvist, P., D. Asrat, I. Nilsson, E. Tsega, G. L. Olsson, B. Wretlind, and J. Giesecke.** 1996. Age at acquisition of *Helicobacter pylori* infection: comparison of a high and a low prevalence country. Scand J Infect Dis **28:**181-4.

148. **Lindkvist, P., F. Enquselassie, D. Asrat, L. Muhe, I. Nilsson, and J. Giesecke.** 1998. Risk factors for infection with *Helicobacter pylori*--a study of children in rural Ethiopia. Scand J Infect Dis **30:**371-6.

149. **Lindkvist, P., F. Enquselassie, D. Asrat, I. Nilsson, L. Muhe, and J. Giesecke.** 1999. *Helicobacter pylori* infection in Ethiopian children: a cohort study. Scand J Infect Dis **31:**475-80.

150. **Logan, R. P., and D. E. Berg.** 1996. Genetic diversity of *Helicobacter pylori*. Lancet **348:**1462-3.

151. **Lu, Y., T. E. Redlinger, R. Avitia, A. Galindo, and K. Goodman.** 2002. Isolation and genotyping of *Helicobacter pylori* from untreated municipal wastewater. Appl Environ Microbiol **68:**1436-9.

152. **Madan, E., J. Kemp, T. U. Westblom, M. Subik, S. Sexton, and J. Cook.** 1988. Evaluation of staining methods for identifying Campylobacter pylori. Am J Clin Pathol 90:450-3.

153. **Maeda, S., and H. Yoshida.** 2001. [Mechanism of drug resistance in *Helicobacter pylori*]. Nippon Rinsho **59:**367-73.

154. **Mahachai, V., P. Tangkijvanich, N. Wannachai, P. Sumpathanukul, and P. Kullavanijaya.** 1999. CagA and VacA: virulence factors of *Helicobacter pylori* in Thai patients with gastroduodenal diseases. Helicobacter **4:**143-7.

155. **Mahalanabis, D., M. M. Rahman, S. A. Sarker, P. K. Bardhan, P. Hildebrand, C. Beglinger, and K. Gyr.** 1996. *Helicobacter pylori* infection in the young in Bangladesh: prevalence, socioeconomic and nutritional aspects. Int J Epidemiol **25:**894-8.

156. **Maki, H., and K. Murakami.** 1997. Formation of potent hybrid promoters of the mutant llm gene by IS256 transposition in methicillin-resistant Staphylococcus aureus. J Bacteriol **179:**6944-8.

157. **Makristathis, A., E. Pasching, K. Schutze, M. Wimmer, M. L. Rotter, and A. M. Hirschl.** 1998. Detection of *Helicobacter pylori* in stool specimens by PCR and antigen enzyme immunoassay. J Clin Microbiol **36:**2772-4.

158. **Malaty, H. M., D. G. Evans, D. J. Evans, Jr., and D. Y. Graham.** 1992. *Helicobacter pylori* in Hispanics: comparison with blacks and whites of similar age and socioeconomic class. Gastroenterology **103:**813-6.

159. **Malaty, H. M., D. Y. Graham, I. Isaksson, L. Engstrand, and N. L. Pedersen.** 1998. Co-twin study of the effect of environment and dietary elements on acquisition of *Helicobacter pylori* infection. Am J Epidemiol **148:**793-7.

160. **Malaty, H. M., N. D. Logan, D. Y. Graham, and J. E. Ramchatesingh.** 2001. *Helicobacter pylori* infection in preschool and school-aged minority

children: effect of socioeconomic indicators and breast-feeding practices. Clin Infect Dis **32:**1387-92.

161. **Malaty, H. M., V. Paykov, O. Bykova, A. Ross, D. P. Graham, J. F. Anneger, and D. Y. Graham.** 1996. *Helicobacter pylori* and socioeconomic factors in Russia. Helicobacter **1:**82-7.

162. **Malfertheiner, P., F. Megraud, C. O'Morain, A. P. Hungin, R. Jones, A. Axon, D. Y. Graham, and G. Tytgat.** 2002. Current concepts in the management of *Helicobacter pylori* infection--the Maastricht 2-2000 Consensus Report. Aliment Pharmacol Ther **16:**167-80.

163. **Marchetti, M., B. Arico, D. Burroni, N. Figura, R. Rappuoli, and P. Ghiara.** 1995. Development of a mouse model of *Helicobacter pylori* infection that mimics human disease. Science **267:**1655-8.

164. **Matsui, T., Y. Matsukawa, T. Sakai, K. Nakamura, A. Aoike, and K. Kawai.** 1995. Effect of ammonia on cell-cycle progression of human gastric cancer cells. Eur J Gastroenterol Hepatol **7 Suppl 1:**S79-81.

165. **McMahon, B. J., T. W. Hennessy, J. M. Bensler, D. L. Bruden, A. J. Parkinson, J. M. Morris, A. L. Reasonover, D. A. Hurlburt, M. G. Bruce, F. Sacco, and J. C. Butler.** 2003. The relationship among previous antimicrobial use, antimicrobial resistance, and treatment outcomes for *Helicobacter pylori* infections. Ann Intern Med **139:**463-9.

166. **McNamara, D., H. Whelan, H. Hamilton, S. Beattie, and C. O'Morain.** 1999. HpSA: assessment of a new non-invasive diagnostic assay for *Helicobacter pylori* infection in an Irish population. Ir J Med Sci **168:**111-3.

167. **Megraud, F.** 1996. Advantages and disadvantages of current diagnostic tests for the detection of *Helicobacter pylori*. Scand J Gastroenterol Suppl **215:**57-62.

168. **Megraud, F., and H. P. Doermann.** 1998. Clinical relevance of resistant strains of *Helicobacter pylori*: a review of current data. Gut **43 Suppl 1**:S61-5.

169. **Megraud, F., and P. Malfertheiner.** 1996. Treatment of *Helicobacter pylori* infection: summary of a meeting at the Fourth United European Gastroenterology Week, September 20, 1995. Helicobacter **1**:118-21.

170. **Mendall, M. A.** 1997. Transmission of *Helicobacter pylori*. Semin Gastrointest Dis **8**:113-23.

171. **Michetti, P., C. Kreiss, K. L. Kotloff, N. Porta, J. L. Blanco, D. Bachmann, M. Herranz, P. F. Saldinger, I. Corthesy-Theulaz, G. Losonsky, R. Nichols, J. Simon, M. Stolte, S. Ackerman, T. P. Monath, and A. L. Blum.** 1999. Oral immunization with urease and Escherichia coli heat-labile enterotoxin is safe and immunogenic in *Helicobacter pylori*-infected adults. Gastroenterology **116**:804-12.

172. **Mitchell, H., and F. Megraud.** 2002. Epidemiology and diagnosis of *Helicobacter pylori* infection. Helicobacter **7 Suppl 1**:8-16.

173. **Mitchell, H. M., P. Hu, Y. Chi, M. H. Chen, Y. Y. Li, and S. L. Hazell.** 1998. A low rate of reinfection following effective therapy against *Helicobacter pylori* in a developing nation (China). Gastroenterology **114**:256-61.

174. **Mitchell, H. M., Y. Y. Li, P. J. Hu, Q. Liu, M. Chen, G. G. Du, Z. J. Wang, A. Lee, and S. L. Hazell.** 1992. Epidemiology of *Helicobacter pylori* in southern China: identification of early childhood as the critical period for acquisition. J Infect Dis **166**:149-53.

175. **Miwa, H., M. F. Go, and N. Sato.** 2002. *H. pylori* and gastric cancer: the Asian enigma. Am J Gastroenterol **97**:1106-12.

176. **Mobley, H. L. T., G. L. Mendz, and S. L. Hazell (ed.).** 2001. *Helicobacter pylori*: physiology and genetics. ASM Press, Washington, D.C.

177. **Mobley, H. L., M. D. Island, and R. P. Hausinger.** 1995. Molecular biology of microbial ureases. Microbiol Rev **59**:451-80.

178. **Mukhopadhyay, A. K., D. Kersulyte, J. Y. Jeong, S. Datta, Y. Ito, A. Chowdhury, S. Chowdhury, A. Santra, S. K. Bhattacharya, T. Azuma, G. B. Nair, and D. E. Berg.** 2000. Distinctiveness of genotypes of *Helicobacter pylori* in Calcutta, India. J Bacteriol **182**:3219-27.

179. **Nabwera, H. M., J. S. Nguyen-Van-Tam, R. F. Logan, and R. P. Logan.** 2000. Prevalence of *Helicobacter pylori* infection in Kenyan schoolchildren aged 3-15 years and risk factors for infection. Eur J Gastroenterol Hepatol **12**:483-7.

180. **Naficy, A. B., R. W. Frenck, R. Abu-Elyazeed, Y. Kim, M. R. Rao, S. J. Savarino, T. F. Wierzba, E. Hall, and J. D. Clemens.** 2000. Seroepidemiology of *Helicobacter pylori* infection in a population of Egyptian children. Int J Epidemiol **29**:928-32.

181. **Nahar, S., A. K. Mukhopadhyay, R. Khan, M. M. Ahmad, S. Datta, S. Chattopadhyay, S. C. Dhar, S. A. Sarker, L. Engstrand, D. E. Berg, G. B. Nair, and M. Rahman.** 2004. Antimicrobial susceptibility of *Helicobacter pylori* strains isolated in Bangladesh. J Clin Microbiol **42**:4856-8.

182. **Nakajima, S., D. Y. Graham, T. Hattori, and T. Bamba.** 2000. Strategy for treatment of *Helicobacter pylori* infection in adults. II. Practical policy in 2000. Curr Pharm Des **6**:1515-29.

183. **Nessa, J., H. Chart, R. J. Owen, and B. Drasar.** 2001. Human serum antibody response to *Helicobacter pylori* whole cell antigen in an institutionalized Bangladeshi population. J Appl Microbiol **90**:68-72.

184. **Nilsson, C., A. Sillen, L. Eriksson, M. L. Strand, H. Enroth, S. Normark, P. Falk, and L. Engstrand.** 2003. Correlation between cag pathogenicity

island composition and *Helicobacter pylori*-associated gastroduodenal disease. Infect Immun **71:**6573-81.

185. **Nomura, A., G. N. Stemmermann, P. H. Chyou, G. I. Perez-Perez, and M. J. Blaser.** 1994. *Helicobacter pylori* infection and the risk for duodenal and gastric ulceration. Ann Intern Med **120:**977-81.

186. **Novik, A. A., N. L. Denisov, M. L. Gershanovich, K. M. Pozharisskii, and I. V. Gorodokin.** 2002. [*Helicobacter pylori* infection and gastric MALT lymphoma: present-day approaches to research and treatment]. Vopr Onkol **48:**283-91.

187. **Nurgalieva, Z. Z., and D. Y. Graham.** 2003. Pearls and pitfalls of assessing *Helicobacter pylori* status. Dig Liver Dis **35:**375-7.

188. **O'Connor, H. J.** 1999. Review article: *Helicobacter pylori* and gastro-oesophageal reflux disease-clinical implications and management. Aliment Pharmacol Ther **13:**117-27.

189. **Odenbreit, S., B. Wieland, and R. Haas.** 1996. Cloning and genetic characterization of *Helicobacter pylori* catalase and construction of a catalase-deficient mutant strain. J Bacteriol **178:**6960-7.

190. **Ogata, S. K., E. Kawakami, F. R. Patricio, M. Z. Pedroso, and A. M. Santos.** 2001. Evaluation of invasive and non-invasive methods for the diagnosis of *Helicobacter pylori* infection in symptomatic children and adolescents. Sao Paulo Med J **119:**67-71.

191. **Okamura, S., H. Katsuhiko, and T. Satoh.** 2000. Regression of rectal mucosa-associated lymphoid tissue lymphoma unrelated to *Helicobacter pylori*. Ann Intern Med **132:**247.

192. **Owen, R. J.** 1998. Helicobacter--species classification and identification. Br Med Bull **54:**17-30.

193. **Pan, Z. J., D. E. Berg, R. W. van der Hulst, W. W. Su, A. Raudonikiene, S. D. Xiao, J. Dankert, G. N. Tytgat, and A. van der Ende.** 1998. Prevalence of vacuolating cytotoxin production and distribution of distinct vacA alleles in *Helicobacter pylori* from China. J Infect Dis **178:**220-6.

194. **Pan, Z. J., R. W. van der Hulst, M. Feller, S. D. Xiao, G. N. Tytgat, J. Dankert, and A. van der Ende.** 1997. Equally high prevalences of infection with cagA-positive *Helicobacter pylori* in Chinese patients with peptic ulcer disease and those with chronic gastritis-associated dyspepsia. J Clin Microbiol **35:**1344-7.

195. **Pantoflickova, D., and A. L. Blum.** 2001. Antagonist: should we eradicate *Helicobacter pylori* in non-ulcer dyspepsia? Gut **48:**758-9.

196. **Parsonnet, J., G. D. Friedman, N. Orentreich, and H. Vogelman.** 1997. Risk for gastric cancer in people with CagA positive or CagA negative *Helicobacter pylori* infection. Gut **40:**297-301.

197. **Parsonnet, J., G. D. Friedman, D. P. Vandersteen, Y. Chang, J. H. Vogelman, N. Orentreich, and R. K. Sibley.** 1991. *Helicobacter pylori* infection and the risk of gastric carcinoma. N Engl J Med **325:**1127-31.

198. **Passaro, D. J., D. N. Taylor, R. Meza, L. Cabrera, R. H. Gilman, and J. Parsonnet.** 2001. Acute *Helicobacter pylori* infection is followed by an increase in diarrheal disease among Peruvian children. Pediatrics **108:**E87.

199. **Patel, P., M. A. Mendall, S. Khulusi, T. C. Northfield, and D. P. Strachan.** 1994. *Helicobacter pylori* infection in childhood: risk factors and effect on growth. Bmj **309:**1119-23.

200. **Paunio, M., J. Hook-Nikanne, T. U. Kosunen, U. Vainio, M. Salaspuro, J. Makinen, and O. P. Heinonen.** 1994. Association of alcohol consumption and *Helicobacter pylori* infection in young adulthood and early middle age

among patients with gastric complaints. A case-control study on Finnish conscripts, officers and other military personnel. Eur J Epidemiol **10:**205-9.

201. **Peck, B., M. Ortkamp, K. D. Diehl, E. Hundt, and B. Knapp.** 1999. Conservation, localization and expression of HopZ, a protein involved in adhesion of *Helicobacter pylori*. Nucleic Acids Res **27:**3325-33.

202. **Peek, R. M., Jr., S. A. Thompson, J. P. Donahue, K. T. Tham, J. C. Atherton, M. J. Blaser, and G. G. Miller.** 1998. Adherence to gastric epithelial cells induces expression of a *Helicobacter pylori* gene, iceA, that is associated with clinical outcome. Proc Assoc Am Physicians **110:**531-44.

203. **Perez Aldana, L., M. Kato, S. Nakagawa, M. Kawarasaki, T. Nagasako, T. Mizushima, H. Oda, J. Kodaira, Y. Shimizu, Y. Komatsu, R. Zheng, H. Takeda, T. Sugiyama, and M. Asaka.** 2002. The relationship between consumption of antimicrobial agents and the prevalence of primary *Helicobacter pylori* resistance. Helicobacter **7:**306-9.

204. **Perez-Perez, G. I., A. F. Cutler, and M. J. Blaser.** 1997. Value of serology as a noninvasive method for evaluating the efficacy of treatment of *Helicobacter pylori* infection. Clin Infect Dis **25:**1038-43.

205. **Perez-Perez, G. I., B. M. Dworkin, J. E. Chodos, and M. J. Blaser.** 1988. Campylobacter pylori antibodies in humans. Ann Intern Med **109:**11-7.

206. **Peterson, W. L.** 2002. Review article: *Helicobacter pylori* and gastric adenocarcinoma. Aliment Pharmacol Ther **16 Suppl 1:**40-6.

207. **Plebani, M., G. Guariso, P. Fogar, D. Basso, N. Gallo, C. F. Zambon, R. Mozrzymas, M. Celadin, and F. Zacchello.** 1999. Effect of cagA status on the sensitivity of enzyme immunoassay in diagnosing *Helicobacter pylori*-infected children. Helicobacter 4:226-32.

208. **Prazeres Magalhaes, P., D. M. De Magalhaes Queiroz, D. V. Campos Barbosa, G. Aguiar Rocha, E. Nogueira Mendes, A. Santos, P. R. Valle**

Correa, A. M. Camargos Rocha, L. Martins Teixeira, and C. Affonso de Oliveira. 2002. *Helicobacter pylori* primary resistance to metronidazole and clarithromycin in Brazil. Antimicrob Agents Chemother **46:**2021-3.

209. **Pronovost, A. D., S. L. Rose, J. W. Pawlak, H. Robin, and R. Schneider.** 1994. Evaluation of a new immunodiagnostic assay for *Helicobacter pylori* antibody detection: correlation with histopathological and microbiological results. J Clin Microbiol **32:**46-50.

210. **Puspok, A., and G. Oberhuber.** 1999. *Helicobacter pylori* and nonulcer dyspepsia. N Engl J Med **340:**1508-9; author reply 1509-11.

211. **Rahman, M. M., D. Mahalanabis, S. A. Sarker, P. K. Bardhan, J. O. Alvarez, P. Hildebrand, C. Beglinger, and K. Gyr.** 1998. *Helicobacter pylori* colonization in infants and young children is not necessarily associated with diarrhoea. J Trop Pediatr **44:**283-7.

212. **Reed, P. I.** 1996. *Helicobacter pylori* and gastric cancer. Eur J Cancer Prev **5 Suppl 2:**49-55.

213. **Rokkas, T., C. Pursey, E. Uzoechina, L. Dorrington, N. A. Simmons, M. I. Filipe, and G. E. Sladen.** 1987. Campylobacter pylori and non-ulcer dyspepsia. Am J Gastroenterol **82:**1149-52.

214. **Rollan, A., R. Giancaspero, F. Fuster, C. Acevedo, C. Figueroa, K. Hola, M. Schulz, and I. Duarte.** 2000. The long-term reinfection rate and the course of duodenal ulcer disease after eradication of *Helicobacter pylori* in a developing country. Am J Gastroenterol **95:**50-6.

215. **Rothenbacher, D., G. Bode, and H. Brenner.** 2002. History of breastfeeding and *Helicobacter pylori* infection in pre-school children: results of a population-based study from Germany. Int J Epidemiol **31:**632-7.

216. **Samra, Z., H. Shmuely, Y. Niv, G. Dinari, D. J. Passaro, A. Geler, E. Gal, M. Fishman, J. Bachor, and J. Yahav.** 2002. Resistance of *Helicobacter*

pylori isolated in Israel to metronidazole, clarithromycin, tetracycline, amoxicillin and cefixime. J Antimicrob Chemother **49:**1023-6.

217. **Sarker, S. A., D. Mahalanabis, P. Hildebrand, M. M. Rahaman, P. K. Bardhan, G. Fuchs, C. Beglinger, and K. Gyr.** 1997. *Helicobacter pylori*: prevalence, transmission, and serum pepsinogen II concentrations in children of a poor periurban community in Bangladesh. Clin Infect Dis **25:**990-5.

218. **Sarker, S. A., M. M. Rahman, D. Mahalanabis, P. K. Bardhan, P. Hildebrand, C. Beglinger, and K. Gyr.** 1995. Prevalence of *Helicobacter pylori* infection in infants and family contacts in a poor Bangladesh community. Dig Dis Sci **40:**2669-72.

219. **Sharma, A. K., S. Mittal, and S. K. Malvi.** 2000. Association of *Helicobacter pylori* with peptic perforation in Chattisgarh region of India. Trop Gastroenterol **21:**42-3.

220. **Shea, J. E., M. Hensel, C. Gleeson, and D. W. Holden.** 1996. Identification of a virulence locus encoding a second type III secretion system in Salmonella typhimurium. Proc Natl Acad Sci U S A **93:**2593-7.

221. **Shepherd, A. J., C. L. Williams, C. P. Doherty, M. Hossack, T. Preston, K. E. McColl, and L. T. Weaver.** 2000. Comparison of an enzyme immunoassay for the detection of *Helicobacter pylori* antigens in the faeces with the urea breath test. Arch Dis Child **83:**268-70.

222. **Shimoyama, T., S. Fukuda, M. Tanaka, T. Mikami, Y. Saito, and A. Munakata.** 1997. High prevalence of the CagA-positive *Helicobacter pylori* strains in Japanese asymptomatic patients and gastric cancer patients. Scand J Gastroenterol **32:**465-8.

223. **Smoot, D. T., H. L. Mobley, G. R. Chippendale, J. F. Lewison, and J. H. Resau.** 1990. *Helicobacter pylori* urease activity is toxic to human gastric epithelial cells. Infect Immun **58:**1992-4.

224. **Spohn, G., and V. Scarlato.** 1999. Motility of *Helicobacter pylori* is coordinately regulated by the transcriptional activator FlgR, an NtrC homolog. J Bacteriol **181:**593-9.

225. **Sue-Ling, H. M., D. Johnston, I. G. Martin, M. F. Dixon, M. R. Lansdown, M. J. McMahon, and A. T. Axon.** 1993. Gastric cancer: a curable disease in Britain. Bmj **307:**591-6.

226. **Suerbaum, S., J. M. Smith, K. Bapumia, G. Morelli, N. H. Smith, E. Kunstmann, I. Dyrek, and M. Achtman.** 1998. Free recombination within *Helicobacter pylori*. Proc Natl Acad Sci U S A **95:**12619-24.

227. **Sullivan, P. B., J. E. Thomas, D. G. Wight, G. Neale, E. J. Eastham, T. Corrah, N. Lloyd-Evans, and B. M. Greenwood.** 1990. *Helicobacter pylori* in Gambian children with chronic diarrhoea and malnutrition. Arch Dis Child **65:**189-91.

228. **Sutton, P.** 2001. Progress in vaccination against *Helicobacter pylori*. Vaccine **19:**2286-90.

229. **Thies, F. L., H. P. Hartung, and G. Giegerich.** 1998. Cloning and expression of the Campylobacter jejuni lon gene detected by RNA arbitrarily primed PCR. FEMS Microbiol Lett **165:**329-34.

230. **Thijs, J. C., A. A. van Zwet, W. J. Thijs, H. B. Oey, A. Karrenbeld, F. Stellaard, D. S. Luijt, B. C. Meyer, and J. H. Kleibeuker.** 1996. Diagnostic tests for *Helicobacter pylori*: a prospective evaluation of their accuracy, without selecting a single test as the gold standard. Am J Gastroenterol **91:**2125-9.

231. **Thomas, J. E., A. Dale, M. Harding, W. A. Coward, T. J. Cole, and L. T. Weaver.** 1999. *Helicobacter pylori* colonization in early life. Pediatr Res **45:**218-23.

232. **Thomas, J. E., G. R. Gibson, M. K. Darboe, A. Dale, and L. T. Weaver.** 1992. Isolation of *Helicobacter pylori* from human faeces. Lancet **340:**1194-5.

233. **Thyagarajan, S. P., P. Ray, B. K. Das, A. Ayyagari, A. A. Khan, S. Dharmalingam, U. A. Rao, P. Rajasambandam, B. Ramathilagam, D. Bhasin, M. P. Sharma, S. R. Naik, and C. M. Habibullah.** 2003. Geographical difference in antimicrobial resistance pattern of *Helicobacter pylori* clinical isolates from Indian patients: Multicentric study. J Gastroenterol Hepatol **18:**1373-8.

234. **Tomb, J. F., O. White, A. R. Kerlavage, R. A. Clayton, G. G. Sutton, R. D. Fleischmann, K. A. Ketchum, H. P. Klenk, S. Gill, B. A. Dougherty, K. Nelson, J. Quackenbush, L. Zhou, E. F. Kirkness, S. Peterson, B. Loftus, D. Richardson, R. Dodson, H. G. Khalak, A. Glodek, K. McKenney, L. M. Fitzegerald, N. Lee, M. D. Adams, J. C. Venter, and et al.** 1997. The complete genome sequence of the gastric pathogen *Helicobacter pylori*. Nature **388:**539-47.

235. **Toracchio, S., and L. Marzio.** 2003. Primary and secondary antibiotic resistance of *Helicobacter pylori* strains isolated in central Italy during the years 1998-2002. Dig Liver Dis **35:**541-5.

236. **Torres, J.** 2000. [Epidemiologic and clinical aspects of *Helicobacter pylori* infection in children]. Rev Gastroenterol Mex **65:**13-9.

237. **Trevisani, L., S. Sartori, F. Galvani, M. R. Rossi, M. Ruina, C. Chiamenti, and M. Caselli.** 1999. Evaluation of a new enzyme immunoassay for detecting *Helicobacter pylori* in feces: a prospective pilot study. Am J Gastroenterol **94:**1830-3.

238. **Tummuru, M. K., T. L. Cover, and M. J. Blaser.** 1994. Mutation of the cytotoxin-associated cagA gene does not affect the vacuolating cytotoxin activity of *Helicobacter pylori*. Infect Immun **62:**2609-13.

239. **Tytgat, G. N., L. A. Noach, and E. A. Rauws.** 1993. *Helicobacter pylori* infection and duodenal ulcer disease. Gastroenterol Clin North Am **22:**127-39.

240. **Vaira, D., P. Malfertheiner, F. Megraud, A. T. Axon, M. Deltenre, A. M. Hirschl, G. Gasbarrini, C. O'Morain, J. M. Garcia, M. Quina, and G. N. Tytgat.** 1999. Diagnosis of *Helicobacter pylori* infection with a new non-invasive antigen-based assay. HpSA European study group. Lancet **354:**30-3.

241. **Vakil, N. B.** 2002. Review article: gastro-oesophageal reflux disease and *Helicobacter pylori* infection. Aliment Pharmacol Ther **16 Suppl 1:**47-51.

242. **van der Ende, A., Z. J. Pan, A. Bart, R. W. van der Hulst, M. Feller, S. D. Xiao, G. N. Tytgat, and J. Dankert.** 1998. cagA-positive *Helicobacter pylori* populations in China and The Netherlands are distinct. Infect Immun **66:**1822-6.

243. **van der Hulst, R. W., J. J. Keller, E. A. Rauws, and G. N. Tytgat.** 1996. Treatment of *Helicobacter pylori* infection: a review of the world literature. Helicobacter **1:**6-19.

244. **Van Doorn, L. J., C. Figueiredo, F. Megraud, S. Pena, P. Midolo, D. M. Queiroz, F. Carneiro, B. Vanderborght, M. D. Pegado, R. Sanna, W. De Boer, P. M. Schneeberger, P. Correa, E. K. Ng, J. Atherton, M. J. Blaser, and W. G. Quint.** 1999. Geographic distribution of vacA allelic types of *Helicobacter pylori*. Gastroenterology **116:**823-30.

245. **van Doorn, L. J., C. Figueiredo, R. Rossau, G. Jannes, M. van Asbroek, J. C. Sousa, F. Carneiro, and W. G. Quint.** 1998. Typing of *Helicobacter pylori* vacA gene and detection of cagA gene by PCR and reverse hybridization. J Clin Microbiol **36:**1271-6.

246. **Van Oijen, A. H., A. L. Verbeek, J. B. Jansen, and W. A. De Boer.** 2000. Review article: treatment of *Helicobacter pylori* infection with ranitidine

bismuth citrate- or proton pump inhibitor-based triple therapies. Aliment Pharmacol Ther **14:**991-9.

247. **Versalovic, J.** 2003. *Helicobacter pylori*. Pathology and diagnostic strategies. Am J Clin Pathol **119:**403-12.

248. **Weaver, L. T., A. J. Shepherd, C. P. Doherty, K. E. McColl, and C. L. Williams.** 1999. *Helicobacter pylori* in the faeces? Qjm **92:**361-4.

249. **Wirth, H. P., M. H. Beins, M. Yang, K. T. Tham, and M. J. Blaser.** 1998. Experimental infection of Mongolian gerbils with wild-type and mutant *Helicobacter pylori* strains. Infect Immun **66:**4856-66.

250. **Wosten, M. M., E. E. Ishiguro, and B. A. van der Zeijst.** 1997. Cloning and characterization of the lytB gene of Campylobacter jejuni. FEMS Microbiol Lett **157:**117-21.

251. **Wu, H., X. D. Shi, H. T. Wang, and J. X. Liu.** 2000. Resistance of *Helicobacter pylori* to metronidazole, tetracycline and amoxycillin. J Antimicrob Chemother **46:**121-3.

252. **Xiang, Z., S. Censini, P. F. Bayeli, J. L. Telford, N. Figura, R. Rappuoli, and A. Covacci.** 1995. Analysis of expression of CagA and VacA virulence factors in 43 strains of *Helicobacter pylori* reveals that clinical isolates can be divided into two major types and that CagA is not necessary for expression of the vacuolating cytotoxin. Infect Immun **63:**94-8.

253. **Xu, Q., R. D. Morgan, R. J. Roberts, and M. J. Blaser.** 2000. Identification of type II restriction and modification systems in *Helicobacter pylori* reveals their substantial diversity among strains. Proc Natl Acad Sci U S A **97:**9671-6.

254. **Yamaoka, Y., M. Kita, T. Kodama, S. Imamura, T. Ohno, N. Sawai, A. Ishimaru, J. Imanishi, and D. Y. Graham.** 2002. *Helicobacter pylori* infection in mice: Role of outer membrane proteins in colonization and inflammation. Gastroenterology **123:**1992-2004.

255. **Yamaoka, Y., D. H. Kwon, and D. Y. Graham.** 2000. A M(r) 34,000 proinflammatory outer membrane protein (oipA) of *Helicobacter pylori*. Proc Natl Acad Sci U S A **97**:7533-8.

256. **Yamaoka, Y., E. Orito, M. Mizokami, O. Gutierrez, N. Saitou, T. Kodama, M. S. Osato, J. G. Kim, F. C. Ramirez, V. Mahachai, and D. Y. Graham.** 2002. *Helicobacter pylori* in North and South America before Columbus. FEBS Lett **517**:180-4.

257. **Zambon, C. F., D. Basso, F. Navaglia, G. Germano, N. Gallo, M. Milazzo, E. Greco, P. Fogar, S. Mazza, F. Di Mario, G. Basso, M. Rugge, and M. Plebani.** 2002. *Helicobacter pylori* virulence genes and host IL-1RN and IL-1beta genes interplay in favouring the development of peptic ulcer and intestinal metaplasia. Cytokine **18**:242-51.

Appendix I & II

Questionnaire of *H. pylori* study

Reg. No: Date: Hospital: DMCH/ICDDR ,B

Name: Age: Years: Sex: M/F

Address:

A) Exclusion criteria:

Concomitant disease Y/N Regular intake of NSAID/Steroid Duration

Treatment in last month Omeprazole /Bisthmuth/ Antibiotic

Pregnant/breast feeding/planning for pregnancy

B) Socio-economic condition

Income Lower (<TK.2500) Middle (TK2500-500) Higher (>TK.5000)

Education Illiterate/Schooling/Higher secondary/above

Smoking habit Y/N Duration: years stick /day

Tobacco/Jarda Y/N Duration: years

Alcohol

Drugs in last 2 m Metronidazole/Antacid/H2 blocker Full course /Occasional

F/history Y/N Ulcer/Gas. Cancer Father/mother/sister/brother/children

C) Clinical presentations

Pain Y/N Abdominal/epigastric Duration Years

Periodicity Nocturnal/day/no relation

Radiation Y/N Back/neck/whole abdomen/no relation

Acid erectation Y/N 1/2/3/4/ Times Duration m After food/evening

Relation with food aggravated/relived/unrelated

Relation with H_2 blocker aggravated/relived/unrelated

D) Abdominal discomfort other than pain

Heart burn	Y/N	Duration	m/	years
Flatulent dyspepsia	Y/N	Duration	m/	years
Anorexia	Y/N	Duration	m/	years
Nausea	Y/N	Duration	m/	years
Vomiting	Y/N	1/2/3/4/day	m/	Auto/induced
UGI bleeding	Y/N	Duration	m/	years
Black tarry stool	Y/N	1/2/3/4/times		days

E) Endoscopy specimens

1) Culture 2) RUT 3) PCR 4) Histopathology 5) Cryosection

F) Endoscopy report

Signature of Endoscopist

G) Collection of blood samples Y/N

H) RUT Start Time: End time:

Positive/Negative Time of positivity min/hours

I) Culture report

J) Histology report

L) Molecular diagnosis

ureC gene Pos./Neg.

cagA gene Pos./Neg.

vacA gene Pos./Neg.

Signature of interviewer

Formation of different media used in this study

Stuart transport media (Merck, Germany)

Composition	Gram/Litre
Sodium glycerophosphate	10.0
Sodium thioglycolate	1.0
Calcium chloride	0.1
Methylene blue	0.002
Agar	8.0

Final volume made up to 1 litre with distilled water pH adjusted to 7.4.

Autoclaved at 121 °C for 20 minutes at 15 Ibs/sq.cm pressure

Bacto Brain Heart Infusion (BD) Gram/litre

Calf Brains, Infusion from200 g	7.7g
Beef Heart, Infusion from 250 g	9.8 g
Protease peptone	10.0g
Dextrose	2.0g
Sodium Chloride	5.0 g
Disodium phosphate	2.5g

37 gram of the powder in 1 liter of purified water, thoroughly mixed and autoclave at 121°C for 15 min.

Urea agar base (oxoid, England)

Composition	Gram/liter
Peptone	1.0
Glucose	1.0
Sodium chloride	5.0
Disodium phosphate	1.2
Potassium dihydrogen phosphate	0.8
Phenol red	0.012
Agar (difco)	15.0

Urea agar base 2.4 gm was dissolved in 90 ml distilled water. Then it was sterilized by autoclaving at 115° C for 20 minutes and cooled to 50°C. Then 10.0ml of sterile 20% urea solution was aseptically introduced to it. Distributed 4ml amounts into sterile container and allowed to set in the slope position.

Solutions that are used in Gel electrophoresis

10X TBE

Composition	Gram/liter
Trizma Base	108.0 g
Boric acid	55.0 g
EDTA	9.3 g
Deionized water	1000 ml

Solution 1 [pH 8.0]

Nacl	100mM
Tris-HCL	10mm
EDTA	10mM

Phosphate buffer saline (PBS) gram/liter

Nacl	89.0 g
Na_2HPO_4	12.0 g
NaH_2PO_4	3.4 g

Final volume made up to 1 liter with distilled water and pH adjusted to 7.2

Helicobacter pylori Selective supplement (Dent)(oxoid)

A freeze-dried selective supplement for the isolation of *Helicobacter pylori*.
Vial contents

Vancomycin	5.0mg
Trimethoprim lactate	2.5mg
Cefsulodin	2.5mg
Amphotericin B	2.5mg

Aseptically 2.0 ml sterile water was added to one vial. When media cooled to 50 °C Dent was added, one vial for 500 ml of media.

Reagents for SDS PAGE

Lower gel buffer

Tris base	181.7g
SDS	4.0g

In 750 ml deionized water, adjust pH to 8.8 with HCl, and then make to 1000ml water. Stored at 4 °C.

Upper gel buffer

Tris base	30.3g
SDS	2.0g

To 350ml of H_2O adjust ph 6.8 with HCl, then make up to 500ml, stored at 4 °C.

30% acryl amide

Acryl amide	300.0g
Bis-acrylamide	8.2g

In 1027ml of deionized water, after dissolve filter through what man #1, stored at 4 °C in brown bottle.

10% ammonium per sulfate

Ammonium per sulfate 10 gram in 100ml of deionized water. Aliquot 100 µl in small amount and stored at –20°C.

Sample buffer

Tris base	7.57g
Glycerol	100ml
2mercaptoethanol	50ml
SDS	23ml

In 750 ml H_2O, adjusted to pH6.8 with HCl, then final volume up to 1 liter, stored at –20 °C.

SDS running buffer

Tris base	30.0g
Glycine	144.0g
SDS	10g

Volume up to 1 liter with H_2O The pH should be 8.2-8.5 not to be adjusted. Stored at 4 °C.

Transfer Buffer

Tris base	3.03g
Glycine	14.40g
Absolute methanol	200.0ml

Volume adjusted to with H_2O to 1 liter. Methanol was added in the buffer after dilution to make 20%.

PBS for Blot

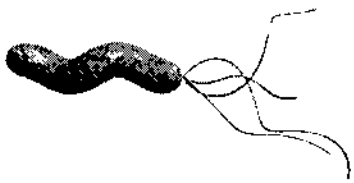
Nacl	8.0g
KCl	0.20g
Na_2HPO_4	1.15g
KH_2PO_4	0.20g

Volume was adjusted to 1liter with deionized water.

TBS 2X

Tris base	4.85g
Nacl	58.0g

After pH adjusted to 7.5,volume was adjusted to 1 liter.



List of Publications

List of Publications

1. Nessa K, Waris SA, Alam A, Huq M, **Nahar S**, Chawdhury FA, Monira S, Badal MU,Sultana J, Mahmud KF, Das J, Mitra DK, Sultan Z, Hossain N, Rahman M. Sexually transmitted infections among brothel-based sex workers in bangladesh: high prevalence of a symptomatic infection.Sex Transm Dis. 2005 Jan; 32(1):13-9.
2. Sarker SA, **Nahar S**, Rahman M, Bardhan PK, Nair GB, Beglinger C, Gyr N. High prevalence of *cagA* and *vacA* seropositivity in a symptomatic Bangladeshi child with *Helicobacter pylori* infection. Acta Paediatr. 2004 Nov; 93(11): 1432-6.
3. **Nahar S**, Mukhopadhyay AK, Khan R, Ahmad MM, Datta S, Chattopadhyay S, Dhar SC, Sarker SA, Engstrand L, Berg DE, Nair GB, Rahman M. Antimicrobial susceptibility of *Helicobacter pylori* strains isolated in Bangladesh. J Clin Microbiol. 2004 Oct; 42(10): 4856-8.
4. Khan R, **Nahar S,** Sultana J, Ahmad MM, Rahman M. T2182C mutation in 23S rRNA is associated with clarithromycin resistance in *Helicobacter pylori* isolates obtained in Bangladesh.Antimicrob Agents Chemother. 2004 Sep; 48(9): 3567-9.
5. Nessa K, Waris SA, Sultan Z, Monira S, Hossain M, **Nahar S,** Rahman H, Alam M, Baatsen P, Rahman M. Epidemiology and etiology of sexually transmitted infection among hotel-based sex workers in Dhaka, Bangladesh. J Clin Microbiol. 2004 Feb; 42(2): 618-21.
6. Sultan Z, **Nahar S,** Wretlind B, Lindback E, Rahman M. Comparison of mismatch amplification mutation assay with DNA sequencing for characterization of fluoroquinolone resistance in Neisseria gonorrhoeae. J Clin Microbiol. 2004 Feb; 42(2): 591-4.
7. Rahman M, Mukhopadhyay AK, **Nahar S**, Datta S, Ahmad MM, Sarker S, Masud IM, Engstrand L, Albert MJ, Nair GB, Berg DE. DNA-level characterization of *Helicobacter pylori* strains from patients with overt disease and with benign infections in Bangladesh. J Clin Microbiol. 2003 May; 41(5): 2008-14. Erratum in: J Clin Microbiol. 2003 Jul; 41(7): 3465.
8. Rahman M, Monira S, **Nahar** S, Ansaruzzaman M, Alam **K**, Alam M, Albert MJ. TnphoA mutants of Providencia alcalifaciens with altered invasiveness of HEp-2 cells. J Med Microbiol. 2002 Aug; 51(8): 682-6.

9. Rahman M, Sultan Z, Monira S, Alam A, Nessa K, Islam S, **Nahar S**, Shama-A-Waris, Alam Khan S, Bogart's J, Islam N, Albert J. Antimicrobial susceptibility of Neisseria gonorrhoeae isolated in Bangladesh (1997 to 1999): rapid shift to fluoroquinolone resistance. J Clin Microbiol. 2002 Jun; 40(6): 2037-40.
10. Bogaerts J, Ahmed J, Akhter N, Begum N, Rahman M, **Nahar S**, Van Ranst M, Verhaegen J. Sexually transmitted infections among married women in Dhaka, Bangladesh: unexpected high prevalence of herpes simplex type 2 infections. Sex Transm Infect. 2001 Apr; 77(2): 114-9.
11. Rahman M, Alam A, Nessa K, **Nahar S**, Dutta DK, Yasmin L, Monira S, Sultan Z, Khan SA, Albert MJ. Treatment failure with the use of ciprofloxacin for gonorrhea correlates with the prevalence of fluoroquinolone-resistant Neisseria gonorrhoeae strains in Bangladesh. Clin Infect Dis. 2001 Mar 15; 32(6): 884-9. Epub 2001 Mar 9.
12. Rahman M, Alam A, Nessa K, Hossain A, **Nahar S**, Datta D, Alam Khan S, Amin Mian R, Albert MJ. Etiology of sexually transmitted infections among street-based female sex workers in Dhaka, Bangladesh. J Clin Microbiol. 2000 Mar; 38(3): 1244-6.
13. Ansaruzzaman M, Albert MJ, **Nahar S**, Byun R, Katouli M, Kuhn I, Mollby R. Clonal groups of enteropathogenic Escherichia coli isolated in case-control studies of diarrhoea in Bangladesh. J Med Microbiol. 2000 Feb; 49(2): 177-85.
14. Ansaruzzaman M, Shimada T, Bhuiyan NA, **Nahar S**, Alam K, Islam MS, Albert MJ. Cross-reaction between a strain of Vibrio mimicus and V. cholerae O139 Bengal. J Med Microbiol. 1999 Sep; 48(9): 873-7.
15. Bhuiyan BU, Rahman M, Miah MR, **Nahar S**, Islam N, Ahmed M, Rahman KM, Albert MJ. Antimicrobial susceptibilities and plasmid contents of Neisseria gonorrhoeae isolates from commercial sex workers in Dhaka, Bangladesh: emergence of high-level resistance to ciprofloxacin. J Clin Microbiol. 1999 Apr; 37(4): 1130-6.
16. Albert MJ, Bhuiyan NA, Talukder KA, Faruque AS, **Nahar S**, Faruque SM, Ansaruzzaman M, Rahman M. Phenotypic and genotypic changes in Vibrio cholerae O139 Bengal. J Clin Microbiol. 1997 Oct; 35(10): 2588-92.
17. Albert MJ, Islam D, **Nahar S**, Qadri F, Falklind S, Weintraub A. Rapid detection of Vibrio cholerae O139 Bengal from stool specimens by PCR. J Clin Microbiol. 1997 Jun; 35(6): 1633-5.

18. Albert MJ, Bhuiyan NA, Rahman A, Ghosh AN, Hultenby K, Weintraub A, **Nahar S**, Kibriya AK, Ansaruzzaman M, Shimada T. Phage specific for Vibrio cholerae O139 Bengal. J Clin Microbiol. 1996 Jul; 34(7): 1843-5.
19. Albert MJ, Ansaruzzaman M, Shimada T, Rahman A, Bhuiyan NA, **Nahar** S, Qadri F, Islam MS. Characterization of Aeromonas trota strains that cross-react with Vibrio cholerae O139 Bengal. J Clin Microbiol. 1995 Dec; 33(12): 3119-23.
20. Albert MJ, Ansaruzzaman M, Qadri F, Hossain A, Kibriya AK, Haider K, **Nahar S**, Faruque SM, Alam AN. Characterisation of Plesiomonas shigelloides strains that share type-specific antigen with Shigella flexneri 6 and common group 1 antigen with Shigella flexneri spp. and Shigella dysenteriae 1. J Med Microbiol. 1993 Sep; 39(3): 211-7.
21. Haider K, Hossain A, Wanke C, Qadri F, Ali S, **Nahar S**. Production of mucinase and neuraminidase and binding of Shigella to intestinal mucin. J Diarrhoeal Dis Res. 1993 Jun; 11(2): 88-92.
22. Albert MJ, Alam K, Ansaruzzaman M, Islam MM, Rahman AS, Haider K, Bhuiyan NA, **Nahar S**, Ryan N, Montanaro J, et al. Pathogenesis of Providencia alcalifaciens-induced diarrhea. Infect Immun. 1992 Dec; 60(12): 5017-24.
23. Haider K, Faruque SM, Albert MJ, **Nahar S**, Neogi PK, Hossain A. Comparison of a modified adherence assay with existing assay methods for identification of enteroaggregative Escherichia coli. J Clin Microbiol. 1992 Jun; 30(6): 1614-6.
24. Faruque SM, Haider K, Albert MJ, Ahmad QS, Alam AN, **Nahar S**, Tzipori S. A comparative study of specific gene probes and standard bioassays to identify diarrhoeagenic Escherichia coli in paediatric patients with diarrhoea in Bangladesh. J Med Microbiol. 1992 Jan; 36(1): 37-40.
25. Haider K, Faruque SM, Shahid NS, Albert MJ, **Nahar S**, Malek A, Tzipori S, Alam AN. Enteroaggregative Escherichia coli infections in Bangladeshi children: clinical and microbiological features. J Diarrhoeal Dis Res. 1991 Dec; 9(4): 318-22.
26. Haider K, Albert MJ, Hossain A, **Nahar S**. Contact-haemolysin production by entero-invasive Escherichia coli and shigellae. J Med Microbiol. 1991 Dec; 35(6): 330-7.
27. Haider K, Albert MJ, **Nahar S**, Kibriya AK. Plasmid-associated resistance to pivmecillinam in Shigella flexneri and Shigella boydii. J Antimicrob Chemother. 1991 Oct; 28(4): 599-601.

28. Albert MJ, Haider K, **Nahar** S, Kibriya AK, Hossain MA. Multiresistant Salmonella typhi in Bangladesh. J Antimicrob Chemother. 1991 Apr; 27(4): 554-5.
29. Haider K, Azad AK, Qadri F, **Nahar S,** Ciznar I. Role of plasmids in virulence-associated attributes and in O-antigen expression in Shigella dysenteriae type 1 strain. J Med Microbiol. 1990 Sep; 33(1): 1-9.

Abstract

1. Haider K, **Nahar** S, Qadri F, Ali S. Association of extra cellular hydrolytic enzymes with virulence in Shigella species. In Proceedings of the 5 th Asian Conference on Diarrhoeal Diseases, 21-23 September. 1989: 53.
2. Haider K, Faruque SM, **Nahar S**, Albert MJ, Shahid NS, Alam AN, Malek A, Tzipori S, Clinical and microbiological features of enteroaggregative Escherichia coli infection in Bangladeshi diarrhoeal children. In: Proceedings of 1st International Scientific Conference of Bangladesh College of Physicians and Surgeons, 22-24 January 1991, Dhaka. Abstract #105.
3. Faruque SM, Haider K, **Nahar S,** Baqui AH, Ahmed QS. Identification of enteroadherent Escherichia coli in Bangladesh diarrhoeal children using specific DNA probes and HeLa cell adherence assay: a comparative study. In: Proceedings of the 8th FAOB Symposium, Dhaka.
4. Haider K, Faruque SM, Hossain A, Baqui AH, Albert MJ, **Nahar S,** Bhuiyan **NA**, Sack RB. Production of protease and hemolysin. Possible markers of virulence in Escherichia coli. Strains isolated from children with diarrhoea. Second Commonwealth Conference on Diarrhoea and Malnutrition, New Delhi, 29th Nov-1st Dec 1991.
5. Albert MJ, Bhuiyan NA, **Nahar S,** Buyn **R**, Alam K, Ansaruzzaman M and Rahman M. Phenotypic changes in *V. Cholerae* O139 Bengal. The Thirty second joint conference US-JAPAN Cooperative Medical Science Programme cholera and related diarrheal diseases panel. Held in Nagasaki University Pompe Hall, Nagasaki, November 14-16, 1996.