Evaluation of *Helicobacter pylori* stool antigen detection test

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TO WHOM IT MAY CONCERN

This is to declare that the research work embodying the results reported in this thesis entitled **″ Evaluation of *Helicobacter Pylori* stool antigen detection test″** submitted by Salma khatun, has been carried out in the laboratory of IMC, BIRDEM during the period of July, 2012 to February, 2014 by the undersigned under the joint supervision of Professor Dr. Naiyyum Choudhury, Co-ordinator, Biotechnology and Microbiology program, Department of Mathematics and Natural Sciences (MNS), BRAC university and Dr. Jalaluddin Ashraful Haq, Professor of Microbiology Dept. & Principal, Ibrahim Medical College & BIRDEM and co-supervision of Dr. Khandaker Shadia**,** Assistant Professor, Department of Microbiology, Ibrahim Medical College & BIRDEM. It is further declared that the research work presented here is original and submitted in partial fulfillment of the requirement for the degree of Masters of Science (M. Sc) in biotechnology under the Department of Mathematics and Natural Sciences (MNS), BRAC University, Dhaka and has not been submitted anywhere else for a degree or diploma. The findings of the research has not been/ will not be published anywhere without the concurrence of the concerned supervisors.

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LIST OF ABBREVIATIONS

H. pylori	*Helicobacter pylori*
NHANES	National Health and Nutrition Examination Survey
MALT	Mucosa-Associated Lymphoid Tissue
RUT	Rapid Urease Test
UBT	Urea Breath Tests
HpSA	*H. pylori* Stool Antigen Assay
FDA	Food and Drug Administration
DNA	Deoxyribonucleic acid
PCR	Polymerase Chain Reaction
ICT	Immune-Chromatographic Test
GIT	Gastro Intestinal Tract
PAI	Pathogenicity Island
Vac A	Vacuolating Cytotoxin A
kDa	KiloDalton
Ure A	Urease A
Ure B	Urease B
Ure C	Urease C
Bab A	Blood group antigen-binding adhesion A
Bab B	Blood group antigen-binding adhesion B
HeBPs	Heparan sulfate binding proteins
SALs	Sialic acid-specific adhesins or lectins
LPS	Lipopolysaccharide
Hsp	Heat shock proteins
IARC	International Agency for Research on Cancer
NH_3	Ammonia
CO_2	Carbon Di Oxaide
ATP	Adenosin Tri Phosphate
OMPs	Outer Membrane Proteins
OipA	Outer membrane inflammatory protein A
MMP-1	Matrix Metalloproteinase 1
GSK-3β	Glycogen Synthase Kinase 3β

T4SS	Type IV secretion system
VacA	Vacuolating Cytotoxin A
IL-8	Interleukin-8
NSAID	Non Steroidal Inflammatory Drug
GERD	Gastro-esophageal reflux disease
CLO	Camphylobacter like Organism
H&E	Haematoxylin & Eosin
ELISA	Enzyme Linked Immune-Sorbent Assay
GHPD	Gastrointestinal, Hepatobiliary and Pancreatic Diseases
BIRDEM	Bangladesh Institute of Research and Rehabilitation in Diabetics Endocrine and Metabolic Disorder
PPIs	Proton Pump Inhibitors
SCABU	Special Care Baby Unit
%	Percentage
PPV	Positive Predictive Value
NPV	Negative Predictive Value
g	Gram
ml	mililitre
µL	mictrolitre
DW	Distilled Water

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ABSTRACT

Helicobacter pylori (*H. pylori*) is a human pathogen causing chronic gastritis, gastric and duodenal ulcer, gastric carcinoma and dyspepsia. The diagnosis of *H. pylori* is an essential element in the management of these gastrointestinal pathologies. There are several invasive and noninvasive methods for diagnosis of Helicobacter pylori infection. The plethora of diagnostic tests available for the presence of *H. pylori* indicates that none of them is 100% accurate. Invasive methods requiring endoscopic evaluation include bacteriologic culture, histopathological studies, smear examination, rapid urease test or CLO test and molecular methods. Noninvasive approaches include serological testing, stool antigen detection and urea breath testing. The stool antigen test is convenient, non-invasive, and does not require the use of expensive equipment or medical personnel. Furthermore, stool can be collected in a patient's home and transferred to the hospital or laboratory.

The aim of the present study was to evaluate the efficacy of *H. pylori* stool antigen detection test (HpSA) in comparison with RUT and serological test.

The present study was carried out on 92 adult patients suffering from dyspepsia and stool samples were examined from another 31 infants as control. From the patients, gastric biopsy specimens were taken for detection of *H. pylori* infection by rapid urease test (RUT). In addition, stool samples were taken for the detection of *H. pylori* antigen and blood samples for detection of anti- *H. pylori* IgA and IgG antibodies.

Among 92 adult patients undergone endoscopic examination, 19 were found having ulcer and 73 were found having erosion. Out of 19 ulcer cases, RUT positive cases were 10 (52.63%) and among 73 erosion cases, 30 (41.09%) cases were RUT positive. *H. pylori* infection was detected in total 40 (43.48%) cases by RUT. Among 19 ulcer cases, 14 (73.68%) cases were positive for HpSA and 44 (60.27%) cases were positive for HpSA out of 73 erosion cases. A total 58 (63.04%) cases were HpSA positive with a sensitivity and specificity of 100% and 96.77% respectively. Serum anti *H. pylori* IgA and IgG antibodies were present in 58 (63.04%) and 89 (96.74%) cases respectively. Both antibodies were present in 57 (61.96%) cases. Among 40 RUT positive cases, IgG and IgA positive cases were 39 (97.5%) and 26 (65%) respectively and both antibodies were present in 25 (62.5%) cases. IgG and IgA positive cases were 50 (96.15%) and 32 (61.54%) respectively and both antibodies were present in 31 (59.62%) cases out of 52 RUT negative patients. Of 40

RUT positive cases, 39 (97.5%) were stool Ag positive. Among 52 RUT negative cases, 19 (36.53%) were positive for stool Ag. Among 58 stool Ag positive patients, 57 (98.27%) cases were IgG positive and 39 (67.24%) cases were IgA positive and 38 (65.52%) cases were positive for both antibodies. Out of 34 stool Ag negative cases, 32 (94.11%) and 19 (55.88%) cases were positive for IgG and IgA respectively and both antibodies were present in 19 (55.88%) cases. Among 31 infants stool samples examined for *H. pylori* antigen as control, 30 (96.77%) were negative for stool Ag. In 58 stool Ag positive cases, serum IgG and IgA mean titre were (2.09 ± 0.46) and (1.40 ± 1.01) respectively. IgG mean titre was 1.94 ± 0.52 and IgA mean tire was 1.23 ± 0.95 in 34 stoo Ag negative patients. *H. pylori* stool antigen assay (HpSA) gave the highest rate of detection (63.04%), followed by serum antibody detection (61.96 %) and the lowest by RUT (43.48%).

H. pylori stool antigen assay (HpSA) could be used as a routine diagnostic tool for *H. pylori* infection.

INTRODUCTION

Helicobacter pylori formerly known as *Campylobacter pyloridis* then *Campylobacter pylori*, is one of the human pathogens with highest prevalence around the world (Parsonnet *et al.,* 1999). It is a small, spiral-shaped, gram-negative, multi-flagellated, micro-aerophilic bacteria commonly found in the gastric mucosa lining the upper gastrointestinal tract (stomach and duodenum) of humans and animals.

Helicobacter pylori infection is ubiquitous and infects both males and females (Goh, 1997; Newell, 1987). More than half of the world's population is infected with *H. pylori*, which is acquired almost always within the first 5 years of life (Suerbaum and Michetti, 2002). The prevalence of *H. pylori* infection varies widely by geographic area, age, race, ethnicity, and socioeconomic status (Feldman, 2001). The overall prevalence of *H. pylori* infection is strongly correlated with socioeconomic conditions. Rates appear to be higher in developing than in developed countries, with most of the infections occurring during childhood, and they seem to be decreasing with improvements in hygiene practices (Malaty and Graham, 1994). Epidemiological data from developed and developing countries suggest that the prevalence of *H. pylori* infection in children under 10 years resident in developed countries being approximately 0 to 5% compared with 13 to 60% in children resident in developing countries. Over this age an increase in prevalence in the order of 0.5 to 2% per annum is commonly observed (Al-Moagel *et al.,* 1990; Graham *et al.,* 1991; Jones *et al.,* 1986). The prevalence among middle-aged adults is over 80 percent in many developing countries, as compared with 20 to 50 percent in industrialized countries (Rowland *et al.,* 1999).

Like other developing countries, the prevalence of *H. pylori* is very high in Bangladesh. The reported prevalence of *H. pylori* infection in adults is about 90% (Bhuiyan, 2010) and more than 80% children become infected with *H. pylori* by the age of 6-9 years (Mahalanabis *et al.,*1996) and 92% adult population are seropositive for *H. pylori* in Bangladesh (Ahmad *et al.,* 1997).

Helicobacter pylori has become the major pathologic agent in the development of chronic gastritis (Dooley *et al*., 1989; Flocca *et al*., 1987), gastric ulcer, duodenal ulcer, MALT lymphoma and gastric cancer and non-ulcerative dyspepsia (Elta *et al.,* 1991) in human. The International Agency for Research on Cancer (IARC) has classified *H. pylori* as a class 1 carcinogen which is in the same class as cigarette smoke (Goodwin and Worsley, 1993;

Atherton, 1997; Marshall *et al.,* 1990). It may also be a risk factor for pancreatic cancer (Trikudanathan et. al, 2011). Association of *H. pylori* has also been demonstrated with some extra-gastric diseases including several autoimmune diseases (Elseweidy, 2013). *H. pylori* infection can lead to impaired glucose tolerance (Chen and Blaser, 2012; Jeon *et al.,* 2012). Recent data from NHANES found a significant association between *H. pylori* infection and increased A1c levels (Chen and Blaser, 2012). In another study, elderly Latinos with *H. pylori* infection were nearly 3 times more likely to develop diabetes than non-infected individuals (Jeon *et al.,* 2012). In a community based endoscopic survey among adult population, the prevalence of peptic ulcer disease was found to be 15.6% ((Hasan *et al.,* 1987), duodenal ulcer to be 11.9% above the age of 15 years (Enroth *et al.,* 2000) in Bangladesh.

This association between *H. pylori* and gastroduodenal diseases necessitates the proper and timely diagnosis of *H. pylori* infection in dyspeptic patients. Both invasive and non-invasive tests are available for diagnosis. The choice of test depends on the cost (variable in each country), availability, clinical situation, prevalence of infection, pretest probability of infection, and presence of confounding factors (e.g. the use of proton pump inhibitors and antibiotics) that may influence test results.

Invasive tests which require endoscopy for diagnostic or therapeutic evaluation. Invasive tests available in clinical practice include: gastric biopsies for culture, gram stain, histology or rapid urease test (RUT).

H. pylori culture is the gold standard for diagnosis of *H. pylori* infection but generally it is not available in most laboratories because it requires special growth condition and of being fastidious organism.

Histopathological examination can provide important information about morphological features indicating severity of gastritis and evidence for dysplasia. However, the accuracy of histopathological examination may be variable due to density of *H. pylori* and sampling error. The accuracy of this test can be improved by adequate biopsies from the antrum and body of the stomach and by special staining such as a silver staining and the Diff-Quik stain (El-Zimaity *et al.,* 1998). Although histopathology and culture of the organism, which are not

easily and routinely performed, is considered the gold standard for the diagnosis of *H. pylori* infection, we need rapid, accurate and reliable noninvasive methods (Kabir, 2001).

The rapid urease test (RUT) is simple and provides quick results (Granstrom *et al.,* 2008). The presence of urease that is produced by *H. pylori* results in hyhdrolysis of neutral urea to alkaline ammonia, which is then visualized by a change in color of the pH indicator. The RUT has a high sensitivity (95%) and specificity (95%) (Howden and Hunt, 1998), making it an excellent primary diagnostic test.
Any concomitant use of antibiotics however will reduces bacterial load, and may lead to false negative results in rapid urease tests (RUT), urea breath test and histology (Vilaichone *et al.,* 2006). Furthermore, the presence of other microorganisms that produce urease can lead to false-positive results (Mégraud and Lehours, 2007).

The noninvasive tests available in clinical practice include serologic tests, urea breath tests (UBT), and stool antigen tests (HpSA).

Serology is widely used for screening patients for *H. pylori* infection. It has a good sensitivity, it is fast, and relatively inexpensive, but it cannot be used after *H. pylori* treatment (Vaira *et al.,* 2000). The serological tests are simple and cheap. IgM antibody test has not proven to be useful clinically, whereas anti–*H. pylori* IgG and IgA have a better result. Anti–*H. pylori* IgG usually can be detected by 3-4 weeks after infection.

Most serologic tests carries a high sensitivity (~90 to 100%), but variable specificity (under 85-90%). Their positive and negative predicative values depend upon the background prevalence of *H. pylori* infection in the population at risk. In areas where infection is common, a negative result is likely to be a false negative. Conversely, a positive result amongst those in whom *H. pylori* is infrequent is more likely to be a false positive. In developed countries with low prevalence of *H. pylori* infection, a positive serological test signals active infection only about half the time. Hence, serology should be validated locally. Further, antibody tests can remain positive for years after *H. pylori* eradication and have limited value to confirm *H. pylori* infection.

The UBT provides a reliable noninvasive method for detection of *H. pylori* infection with sensitivity and specificity of 88-95% and 95%-100% respectively (Howden and Hunt, 1998). Urea breath test is not only sensitive and specific but also has an important advantage to

confirm *H. pylori* eradication. Following ingestion of 13C- or 14C-urea, *H. pylori*-produced urease enzyme that is resident in the stomach hydrolyzes this labeled urea to $^{14}CO_2$ or $^{13}CO_2$, which can be detected in breath samples (Vaira *et al.,* 1999). The nonradioactive^{13}C (a stable label) test and the radioactive ^{14}C test have received US Food and Drug Administration (FDA) approval for *H. pylori* diagnosis. The dose of radiation in the ^{14}C-urea test however is not approved for use in children and pregnant women (Vilaichone *et al.,* 2006). Moreover, the UBT requires an expensive instrument such as a mass spectrometer, which is not always available in routine clinical laboratories (Hoda *et al.,* 2006).

As a gastrointestinal pathogen, *H. pylori* also appear in the stool. It allows the development of fecal assays like *H. pylori* culture, DNA detected by polymerase chain reaction (PCR), or *H. pylori* stool antigen (HpSA) testing. Among them only stool antigen has proven to be clinically useful with sensitivities and specificities of more than 90%. Stool antigen assay is advantageous to confirm eradication (Vilaichone *et al.,* 2006). It can be used as a routine diagnostic tool for *H. pylori* infection because it seems to overcome the limitations of the conventional invasive techniques. This new immune-chromatographic test (ICT) has the advantage of being non invasive, easy and fast to perform, useful with individuals for whom endoscopy is difficult to justify, to monitor treatment response and cheaper than the invasive and molecular tests. Moreover, HpSA indicates the active infection of the patient with *H. pylori*.

The aim of the present study was to evaluate the efficacy of *H. pylori* stool antigen detection test (HpSA) to diagnose *H. pylori* infection in dyspeptic patients in comparison with traditional techniques.

Objectives:

The Objectives of the present study were to:

- do rapid usease test using antral biopsy material of the dyspeptic patients obtained during upper GIT endoscopy.
- determine anti- *H. pylori* IgA and IgG level in dyspeptic patients.
- detect *H. pylori* antigen from stool samples of the patients.
- detect *H. pylori* antigen from stool samples of the neonates taken as control.

REVIEW OF LITERATURE

Overview

Today's understanding of Helicobacter-related gastric diseases in humans stems from an explosion in research, which occurred after the first culture of the organism by Marshall and Warren in 1982 (Warren and Marshall, 1983). This event may have been the culmination of over 100 years of study of helicobacters and their epiphenomena. The presence of spiral-shaped micro-organisms in stomach mucosa was described almost 100 years ago (Madan *et al.*, 1988). This discovery rapidly revolutionized the discipline of gastroenterology in human medicine and identified it as the causative agent of gastritis and peptic ulcer (Warren and Marshall, 1984).

History

By the late 19th and early 20th centuries, several investigators had reported the presence of spiral microorganisms in the stomachs of animals (Bizzozero, 1893). Soon afterward, similar spiral bacteria were observed in humans (Krienitz**,** 1906; Luger, 1917), some of whom had peptic ulcer disease or gastric cancer. The etiological role of these bacteria in the development of peptic ulcer disease and gastric cancer was considered at that time, and patients were sometimes even treated with high doses of the antimicrobial compound bismuth (Pel, 1899). This possibility was later discarded as irrelevant, probably because of the high prevalence of these spiral bacteria in the stomachs of persons without any clinical signs. The bacteria observed in human stomachs were thus considered to be bacterial overgrowth or food contaminants until the early 1980s. At that time, Warren and Marshall performed their groundbreaking experiments, leading to the identification of a bacterium in 58 of 100 consecutive patients, with successful culture and later demonstration of eradication of the infection with bismuth and either amoxicillin or tinidazole (Marshall *et al*., 1985). The organism was initially named "*Campylobacter*-like organism," "gastric *Campylobacter*-like organism," "*Campylobacter pyloridis*," and "*Campylobacter pylori*" but is now named *Helicobacter pylori* in recognition of the fact that this organism is distinct from members of the genus *Campylobacter* (Goodwin *et al*., 1989). It soon became clear that this bacterium causes chronic active gastritis, which in a subset of subjects may progress to other conditions, in particular, peptic ulcer disease, distal gastric adenocarcinomas and gastric lymphomas (Ernst and Gold, 2000)**.**

Microbiology

Genus Description and Phylogeny

The genus *Helicobacter* belongs to the subdivision of the *Proteobacteria*, order *Campylobacterales*, family *Helicobacteraceae*. This family also includes the genera *Wolinella*, *Flexispira*, *Sulfurimonas*, *Thiomicrospira*, and *Thiovulum*. To date, the genus *Helicobacter* consists of over 20 recognized species, with many species awaiting formal recognition (Fox, 2002). Members of the genus *Helicobacter* are all microaerophilic organisms and in most cases are catalase and oxidase positive, and many but not all species are also urease positive.

Helicobacter species can be subdivided into two major lineages, the gastric *Helicobacter* species and the enterohepatic (nongastric) *Helicobacter* species. Both groups demonstrate a high level of organ specificity, such that gastric helicobacters in general are unable to colonize the intestine or liver, and vice versa. An extensive review of non-pylori *Helicobacter* species is available (Solnick and Schauer, 2001**),** and here we briefly discuss those *Helicobacter* species that are either associated with human disease or have relevance for animal models of human *Helicobacter* infections (Table 2.1).

Table 2.1: Characteristics of selected *Helicobacter* species.

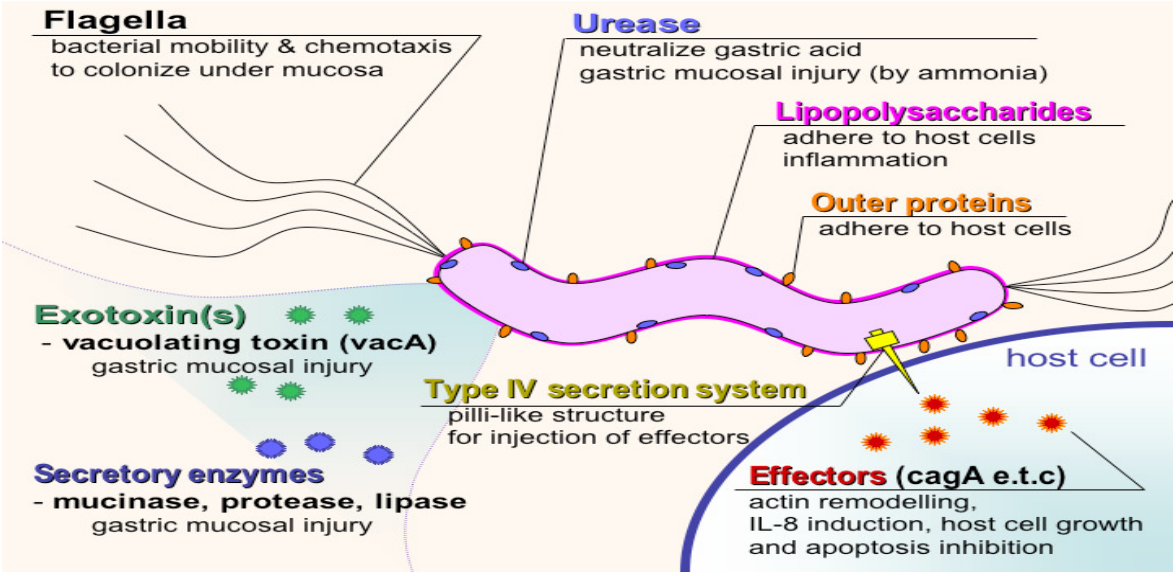
Species	**Primary mammalian host**	**Pathology**	**Animal model**
Gastric *Helicobacter* spp.			
H. pylori	Human, primate	Gastritis, peptic ulcer disease, gastric adenocarcinoma, MALT lymphoma	Mouse, Mongolian gerbil, Guinea pig, Gnotobiotic piglet
H. felis	Cat, dog, mouse	Gastritis in natural host; may cause peptic ulcers or gastric adenocarcinoma in mouse	Mouse

H. mustelae	Ferret	Gastritis, peptic ulcer disease, gastric adenocarcinoma, MALT lymphoma	None
H. acinonychis	Cheetah, tiger, other big cats	Gastritis, peptic ulcer disease	Mouse
H. heilmannii	Human, dog, cat, monkey, cheetah, rat	Gastritis, dyspeptic symptoms, MALT lymphoma	Mouse
Enterohepatic *Helicobacter* spp.			
H. hepaticus	Mouse, other rodents	Proliferative typhlocolitis, hepatitis, hepatocellular carcinoma	None

Microbiological characteristics of *H. pylori*

Helicobacter pylori (*H. pylori*) is a slow-growing, microaerophilic, spiral shaped multi flagellated, gram-negative bacterium, about 3 micrometres long with a diameter of about 0.5 micrometres, whose surface is coated with 12–15 nm ring-shaped aggregates of urease and heat shock protein. The urease enzyme and the heat shock protein B are located almost exclusively within the cytoplasm in the fresh log-phase cultures of *H. pylori*. In subcultures, urease and heat shock protein B become associated with the bacterial surface, suggesting bacterial autolysis leading to release of protein and adsorption into the bacterial surface. Some of the lipopolysaccharide of the organism mimics the Lewis blood group antigens in structure. This molecular mimicry also helps in the continued existence of *H. pylori* in the unfavorable gastric environment. This bacterium colonizes gastric mucosa and elicits both inflammatory and immune lifelong responses, with release of various bacterial and host-dependent cytotoxic substances (Peterson *et al*., 1998). Under unfavorable circumstances it can become coccoidal, a nonculturable form with debatable viability. The bacterium is a microaerophilic and capnophilic organism, slowly growing with rigorous culture demands (Mégraud and Lehours, 2007).

Figure 2.1: Components of *Helicobacter pylori* with biological activity



Genome structure

H. pylori consist of a large diversity of strains, and the genomes of three have been completely sequenced (Tomb *et al*., 1997; Oh *et al*., 2006). The genome of the strain "26695" consists of about 1.7 million base pairs, with some 1,550 genes. The two sequenced strains show large genetic differences, with up to 6% of the nucleotides differing ("*Helicobacter pylori*26695, complete genome, 2008).

Study of the *H. pylori* genome is centered on attempts to understand pathogenesis, the ability of this organism to cause disease. Approximately 29% of the loci are in the "pathogenesis" category of the genome database. Two of sequenced strains have an approximately 40 kb-long Cag pathogenicity island (a common gene sequence believed responsible for pathogenesis) that contains over 40 genes. This pathogenicity island is usually absent from *H. pylori* strains isolated from humans who are carriers of *H. pylori*, but remain asymptomatic (Baldwin *et al*., 2007).

The cagA gene codes for one of the major *H. pylori* virulence proteins. Bacterial strains that have the cagA gene are associated with an ability to cause ulcers (Broutet *et al*., 2001). The cagA gene codes for a relatively long (1186 amino acid) protein. The cag pathogenicity island (PAI) has about 30 genes, part of which code for a complex type IV secretion system. The low GC-content of the cag PAI relative to the rest of the Helicobacter genome suggests

the island was acquired by horizontal transfer from another bacterial species (Tomb *et al*., 1997).

Cell Biology of *H. pylori*

Factors involved in colonization and adhesion

Several virulence factors for gastric colonization, tissue damage, and survival have been identified in *H. pylori* (Table 2.2). Flagella, urease, and adhesins are all essential factors for *H. pylori* to colonize the gastric mucosa. Mutants of *H. pylori* without flagella or without urease are unable to colonize the gastric mucosa in laboratory animals (Eaton *et al*., 1991).

Table 2.2: Virulence factors identified in *H. pylori*

Virulence factor	**Effect**
Colonizing	
Flagella	Active movements through mucin
Urease	Neutralization of acid
Adhesins	Anchoring *H. pylori* to epithelium
Tissue damaging	
Proteolytic enzymes	Glucosulfatase degrades mucin
120-kDa cytotoxin (Gac A)	Related to ulcer and severe gastritis
Vacuolating cytotoxin (Vac A)	Damage of the epithelium
Urease	Toxic effect on epithelial cells, disrupting cell tight junctions
Phospholipase A	Digest phospholipids in cell membranes
Alcohol dehydrogenase	Gastric mucosal injury
Survival	
Intracellular surveillance	Prevent killing in phagocytes
Superoxide dismutase	Prevent phagocytosis and killing
Catalase	Prevent phagocytosis and killing
Coccoid forms	Dormant form
Heat Shock proteins	
Urease	Sheathing antigen
Others	

Lipopolysaccharide	Low biological activity
Lewis X/Y blood group homology	Autoimmunity

Source: Chapter 4, Basic Bacteriology and Culture

Flagella and motility

The curved morphology of *H. pylori* and the polar motility caused by flagella in one end cause screw-like movements, which may enable the organism to penetrate the mucin layer. The motility of *H. pylori* is increased when the viscosity of the media is increased in vitro and transverses a methyl glucose solution 10 times more efficiently than Escherichia coli (Hazell *et al*., 1986), but the motility is pH dependent and impaired at a pH below 4 (Miller-Podraza *et al*., 1999).

Urease, catalase, superoxide dismutase, and alkylhydroperoxidase reductase

Urease is one of the key enzymes in *H. pylori* pathogenesis. It has a molecular weight of 550 kDa and consists of three subunits of 26.5 kDa (Ure A), 61 kDa (Ure B), and 13 kDa (Ure C) (Labigne *et al*., 1991; Mobley *et al*., 1988). Urease is necessary for *H. pylori* to maintain a pH-neutral microenvironment around the bacteria, necessary for survival in the acidic stomach (Hawtin *et al*., 1990; Perez-Perez *et al*., 1992). Urease induces self-destruction of *H. pylori* in vitro in nonacidic media (Neithercut *et al*., 1991). Urease is strongly immunogenic and chemotaxic for phagocytes.

Superoxide dismutase has been isolated from *H. pylori*, which breaks down superoxide produced in polymorphonuclear leukocytes and macrophages and thereby prevents the killing of these organisms (Spigelhalder *et al*., 1993). Catalase protects *H. pylori* against the damaging effects of hydrogen peroxide released from phagocytes (Hazell *et al*., 1991). Urease and catalase may be excreted from *H. pylori* to the surrounding environment and may protect this pathogen from the humoral immune response (Hawtin *et al*., 1990).

Outer membrane proteins, phospholipids, glycolipids, and other adhesins

H. pylori adheres to mucin and binds specifically to gastric mucosa epithelial cells both in vivo and in vitro (Hessay *et al*., 1990; Falk *et al*., 1993; Tzouvelekis *et al*., 1991). Different adhesion patterns, which are different in children and in adults, have been described (Blom *et al*., 2000). Several putative gastric tissue receptor structures have been described for *H. pylori* such as (i) sialoglycoconjugates in gastric mucins and on epithelial cells, phagocytes, and

extra-cellular matrix (Evans *et al*., 1988; Hynes *et al*., 1999; Wadstrom *et al*., 1996), (ii) sulfated glycoconjugates such as heparan sulfate and other glycosaminoglycans (Wadström *et al*., 1999), and (iii) sulfatides (Saitoh *et al*., 1991; Utt and Wadström, 1997) and various sialylated and nonsialylated glycolipids (Miller-Podraza *et al*., 1999).

Binding of *H. pylori* to cell surface fucosylated blood group antigens, H1 and Lewis part of the blood group O in the ABO system, was first described by Borén and collaborators (Borén *et al*., 1993) and was shown to mediate adherence to human and rhesus monkey gastric tissue surface cells (Borén *et al*., 1997). More recently, Peterson and collaborators (Peterson *et al*., 2000) identified the *H. pylori* blood group antigen-binding adhesins, Bab A and Bab B, purified the proteins, and cloned the babA and babB genes. Two closely related basic proteins of 78 kDa were characterized. The sialic acid-specific lectins of 19 and 23 kDa have also been purified and the corresponding genes cloned (T. Borén, personal communication). These proteins, unlike BabA and -B, do not belong to the family of proteins, mostly named helicobacter outer membrane proteins by a nomenclature introduced by T. J. Trust and associates. Cell surface adhesins recognizing sulfatides were not identified, whereas heparan sulfate binding proteins (HeBPs) were purified and characterized by Utt *et al*. (151; unpublished). None of the glycolipid-binding surface proteins has been purified and characterized yet. Interestingly, no similar putative mucin and cell adhesin has been identified in *H. felis* or other newly identified species, except for *H. mustelae* (P. O'Toole and T. Trust, personal communication). In vitro affinity binding studies for the Lewis binding give high-affinity constants (K_a, − 2.5; 10 to 11 M) and also reveal high affinity for human mucin-binding glycoconjugates to hemagglutinating sialic acid-specific adhesins or lectins (SALs) (Wadstrom *et al*., 1996). Recent studies by Syder *et al*. (Syder *et al*., 1999) in a transgene mouse model suggest that SALs may become key adhesins in inflamed gastric mucosa. It is noteworthy that these SALs are produced already in the exponential growth phase while the Bab A and B adhesins appear mainly in the stationary phase cells. SALs and HeBPs may be the key receptors on leukocytes and macrophageys, and trigger lectinophagocytosis as for several other microbial pathogens (Wadstrom *et al*., 1999).

Factors involved in tissue damage and survival factors

Enzymes: protease, etc

Conflicting results have been reported concerning proteolytic enzymes in *H. pylori*. It seems probable that *H. pylori* glycosulfatase degrades gastric mucin (Sidebotham *et al*., 1991; Slomiany *et al*., 1992). *H. pylori* possess phospholipase A, which can digest phospholipids of cell membranes (Langton and Cesareo, 1992). Urease has a cytotoxic activity (Minocha *et al*., 1994). Recently, alcohol dehydrogenase has been described to contribute to gastric mucosal injury (Roine *et al*., 1992).

Toxins: vacuolating cytotoxin A, lipopolysaccharide

Helicobacter pylori contains a toxin, Vac A, which can produce vacuoles in gastric epithelial cells and has been related to peptic ulcer, severe gastritis (Catrenich and Chestnut, 1992; Cover *et al*., 1993), and mucosal integrity (Figura, 1996). Lipopolysaccharide (LPS) in *H. pylori* has a low biological activity as compared to LPS from other gram-negative bacteria (Nielsen, 1994), which may be explained by the unusual composition of lipid A (Mattsby-Baitzer *et al*., 1992). LPS from *H. pylori* stimulates phenotypic transcription and functional changes in monocytes (Nielsen, 1994). Assays using gastric mucosal laminin (integrin) receptor binding to a laminin-coated surface have revealed a significant decrease in receptor binding occurring in the presence of *H. pylori* LPS. When the gastric epithelial barrier is weakened by disruption of the mucosal surface cells and the extracellular matrix, LPS is responsible for a marked increase in epithelial cell apoptosis (Piotrowski *et al*., 1997). LPS from *H. pylori* has recently attracted interest because LPS from most strains mimic Lewis and/or Lewis blood group antigens (Moran *et al*., 1996). This mimicry may play a role in the regulation of the immune response and induce autoantibodies against the gastric proton pump.

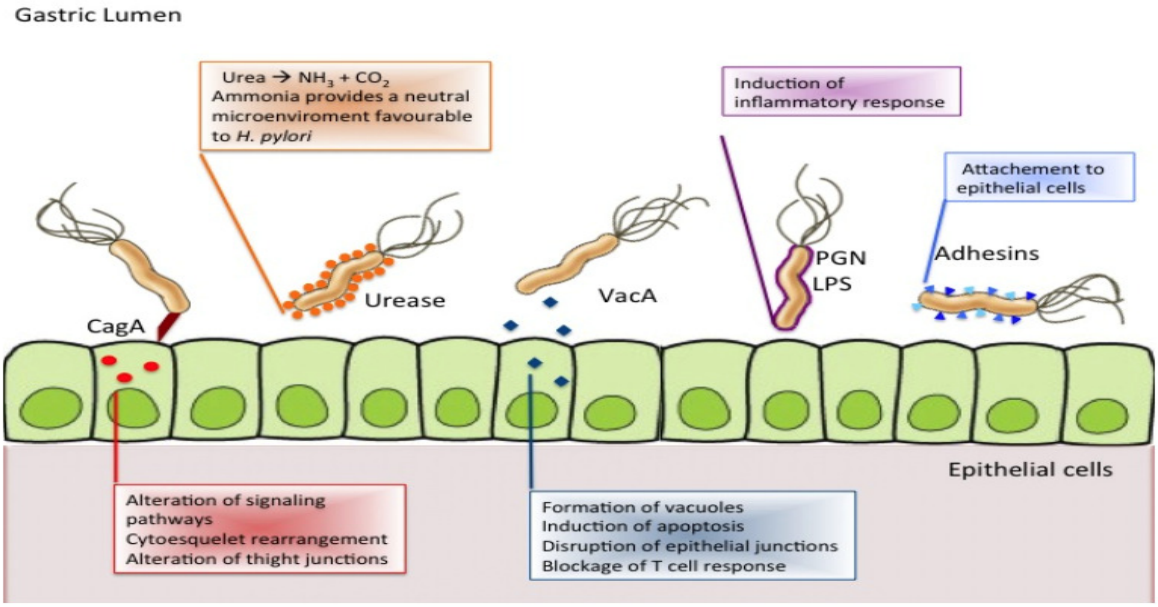
Other putative virulence factors

Several heat shock proteins (Hsp) such as 58.2-kDa Gro-El (Hsp B), 13-kDa Gro-Es (Hsp A), and a 70-kDa Hsp have been identified in *H. pylori* (Moutiala *et al*., 1992; Dunn *et al*., 1992). Hsp are produced by all cells and are involved in stabilizing and probably repairing proteins under harsh conditions that may be important to survive in the stomach.

Helicobacter pylori transforms into coccoid forms (Bode *et al*., 1992; Catrenich and Makin, 1991) under certain conditions such as nutrient starvation and media containing growth

inhibitors (bismuth, proton pump inhibitor, or certain antibiotics). These coccoid forms have been reported to survive for several years in river water and have been proposed by some to be an important factor for transmission, by fecal excretion, and for therapy failure.

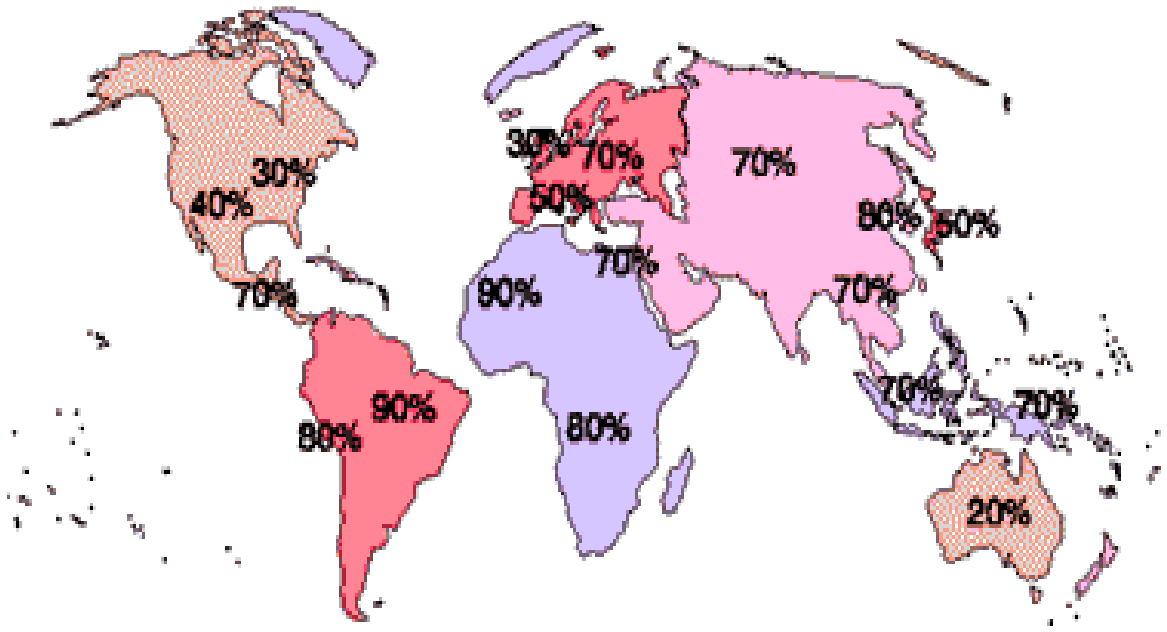
Figure 2.2: *Helicobacter pylori* virulence factors in gastritis and gastric cancer



Epidemiology

Helicobacter pylori is now regarded as the most widespread infection in man: it has a world-wide distribution and it is estimated that approximately two-thirds of the entire world's population is infected with this pathogen (Dunn *et al*., 1990). Most infections are believed to be acquired during childhood and appear to persist for decades (Blaser, 1996). *H. pylori* is known to be the most important causal agent of human gastritis, gastric and duodenal ulcers, and has recently been classified as a first class human carcinogen by IARC (International Agency for Research on Cancer) (Anonymous, 1994).

Figure 2.3: Global prevalence of *H. pylori* infection.



The prevalence of *H. pylori* shows large geographical variations and may differ between different ethnic, social, or age groups within the same country [Malaty, 1996; Goh, 1997; Mitchell, 1992). Globally, the prevalence of *H. pylori* infection in developing countries is markedly higher than that in developed countries (Mégraud *et al*., 1989; Graham *et al*., 1991; Bardhan, 1997; Perez-Perez *et al*., 2004). Moreover, the acquisition of *H. pylori* seems to occur at higher rates in developing countries (Pounder, 1995; Mitchell *et al*., 1992). In various developing countries, more than 80% of the population is *H. pylori* positive, even at young ages (Perez-Perez *et al*., 2004). The prevalence of *H. pylori* in industrialized countries generally remains under 40% and is considerably lower in children and adolescents than in adults and elderly people (pounder, 1995). Within geographical areas, the prevalence of *H. pylori* inversely correlates with socioeconomic status, in particular in relation to living conditions during childhood (Malaty, 1996). In Western countries, the prevalence of this bacterium is often considerably higher among first- and second-generation immigrants from the developing world (Perez-Perez *et al*., 2005; Tsai *et al*., 2005). While the prevalence of *H. pylori* infection in developing countries remains relatively constant, it is rapidly declining in the industrialized world (Genta, 2002). The latter is thought to be caused by the reduced chances of childhood infection due to improved hygiene and sanitation and the active elimination of carrier ship via antimicrobial treatment. In developing countries, *H. pylori*

infection rates rise rapidly in the first 5 years of life and remain constantly high thereafter, indicating that *H. pylori* is acquired early in childhood (Feidorek *et al*., 1991). However, in industrialized countries the prevalence of *H. pylori* infection is low early in childhood and slowly rises with increasing age. This increase results only to a small extent from *H. pylori* acquisition at later age. The incidence of new *H. pylori* infections among adults in the Western world is less than 0.5% per year; the higher prevalence of infection among the elderly thus reflects a birth cohort effect with higher infection rates in the past (Kuipers *et al*., 1995; Parsonnet, 1995). The active elimination of *H. pylori* from the population and improved hygiene and housing conditions have resulted in a lower infection rate in children, which is reflected in the age distribution of this lifelong-colonizing bacterium (Rehnberg *et al*., 2001; Kuipers, 1999). Overall, new infection more commonly occurs in childhood and lasts for life unless specifically treated. A plethora of studies reported and emphasized these differences within and between countries (Table 2.3).

Table 2.3: Prevalence of *H. pylori* infection in different populations of the world

Country	**Number of Studies**	**Age range (Years)**	**Prevalence (%)**	**Reference**
Developing countries[1]				
Bangladesh	181	20-44	92%	Ahmad *et al*., 1997
Brazil				
8u-Rural	40(Children)	<20	77.5%	Souto *et al*., 1998
	164(Adults)	20-90	84.7%	
-Urban	363	>20	63.4%	Santos *et al*., 2005
-Poor urban community	204	18-80	80%	Rodrigues *et al*., 2005
Colombia(rural)	684	2-9	69%	Goodman *et al*., 1996

China				
-South China	1727	N/A	44.2%	Mitchell *et al*., 1992
-Hong Kong	397	36-65	58.6%	Wong *et al*., 1999
Changle of Fujan	1456	-	80.4%	Wong *et al*., 1999
Egypt				
Alexandria (northern)	169 mothers	N/A	88%	Bassily *et al*., 1999
	169 children	< 1	13%	
		1.5	25%	
- Cairo (central)	52	< 6	33%	Frenck *et al*., 2005
	56	> 6	60%	
-Assiut (southern)	Urban	N/A	87%	Sayed *et al*., 2007
	Rural	N/A	40%	
- poor urban area	Schoolchildren	N/A	72.4%	Mohammad *et al*., 2008
India	238	3-70	79%	Graham *et al*., 1991
Mexico	11605	20-90	66%	Torres *et al*., 1998
Nepal (rural)	1142	4-93	56.8%	Kawasaki *et al*., 1998
	407	2 mo-12 yr	48%	Klein *et al*., 1991
Peru	104	0-17	50%	Begue *et al*., 1998
Russia	213	20-75	88%	Malaty *et al*., 1996
- St. Petersburg 1995	307	2-19	44%	Tkachenko *et al*., 2007; Mataly *et al*., 1996

- St. Petersburg 2005	370	2-19	13%	Tkachenko *et al*., 2007
Saudi Arabia	557	5-10	40%	Al-moagel *et al*., 1990
		> 20	70%	
Taiwan	823	1-40+	54%	Teh *et al*., 1994
Developed Countries[1]:				
Australia (urban Melbourne)	273	19-47	23%	Lambert *et al*., 1995
Denmark	3589	30-60	25.9%	Andersen *et al*., 1996
Germany (western)	260	18-61	39.2%	Breuer *et al*., 1996
Israel (rural)	377	30-90	72%	Gilboa *et al*.., 1995
Japan	4361	19-69	30%	Kikuchi *et al*., 1998
Netherlands	254 (employees)	11-89	27.2%	Böhmer *et al*., 1997
New Zealand	579 workers:	40-64	56%	
- Europeans	190		35.8%	
- Maori	195		57.4%	
- Pacific Islanders	194		73.2%	Fraser *et al*., 1996
Spain	332	> 18	43%	Senra-Varela *et al*., 1998
- mountain	178		54%	
- coastal	154		30%	
South Korea	161	20-75	75%	Malaty *et al*., 1996

Switzerland	176 natives	10-20	7.3%	Heuberger *et al*., 2003
	20 immigrants		30%	
United Kingdom				
- England	267 (healthy)	> 18	41%	Harris *et al*., 1995
	467 (all males)	18-65	37.5%	Webb *et al*., 1996
- Northern Ireland	4742	12-64	50.5%	Murry *et al*., 1997
- South Wales	1796	45-59	70%	Strachan *et al*., 1998
United States				
- South Carolina	938 army recruits	17-26	26%	
	324 blacks		44%	Smoak *et al*., 1994
	47 Hispanics		38%	
	536 whites		14%	
- California	556	20-39	27%	Replogle *et al*., 1995
- Texas	246 blacks	15-80	70%	Graham *et al*., 1991
	239 whites	34%		

Source: Khalifa *et al. Gut Pathogens* 2010 **2**:2

1. The classification into developing or developed countries was retrieved from the United Nation Development Programme's Human Development Reports (URL: http://hdr.undp.org/en webcite).

N/A: Data not available or not applicable

Transmission of *H. pylori*

The mode of transmission of *H. pylori* is one of the most controversial areas in the study of this pathogen. The exact mechanisms whereby *H. pylori* is acquired are largely unknown.

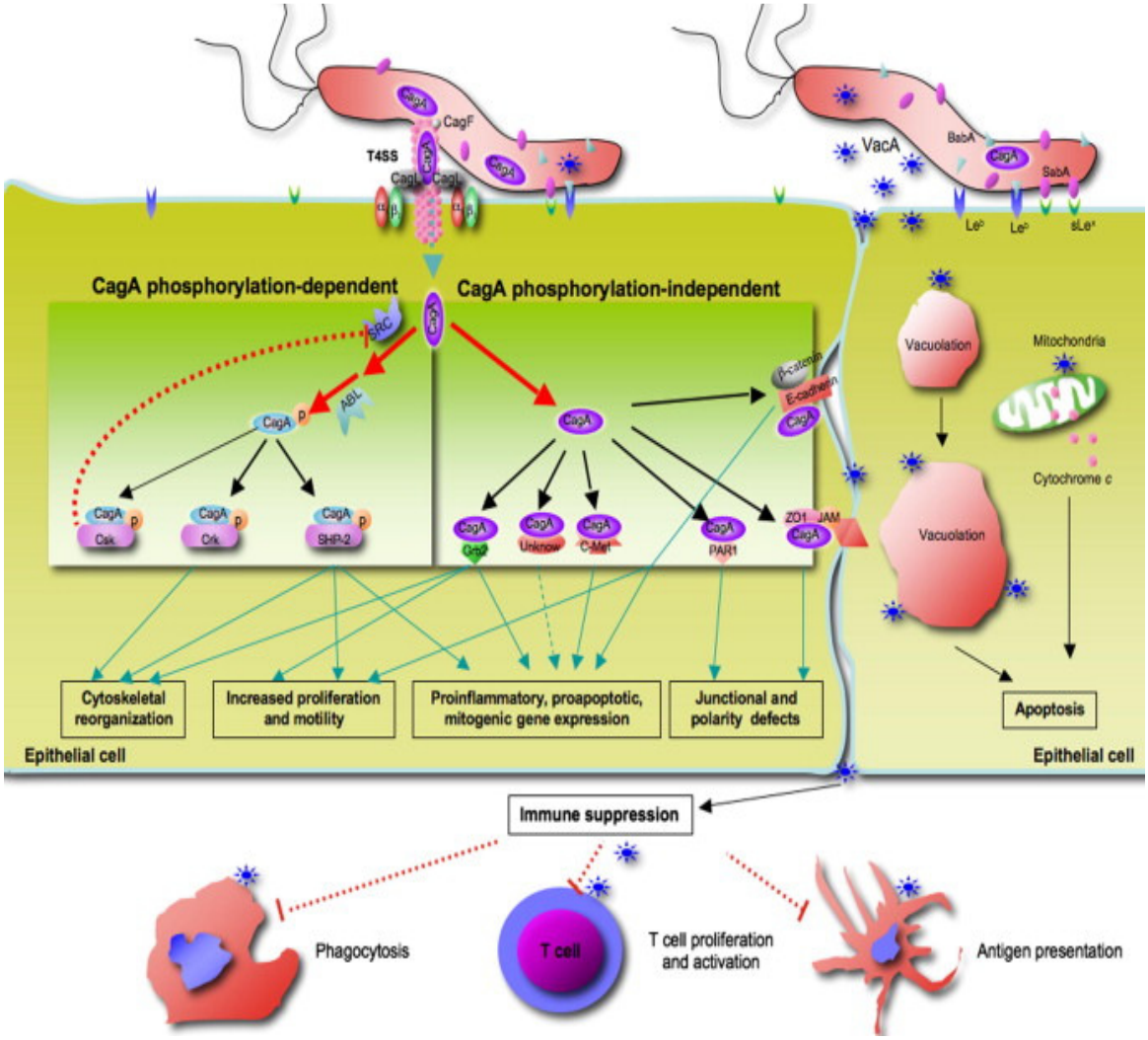
H. pylori has a narrow host range and is found almost exclusively in human and some nonhuman primates. Despite hostile, the human stomach is the only identified reservoir for *H. pylori*. Although extensively studied, efforts to confirm the exact route of transmission have been disappointing. It has been speculated that the person-to-person spread currently appeared to be the most likely mode of transmission, especially between family members (Weyermann *et al*., 2006; Kivi *et al*., 2005). Hence, the possible routes are fecal–oral, oral–oral and gastro–oral (Gold, 2001).Thomas *et al.,* were able to isolate *H. pylori* DNA and culture *H. pylori* from human feces, suggesting a fecal–oral transmission (Thomas *et al*., 1992). Oral–oral transmission has also been recognized because *H. pylori* was isolated from dental plaques and saliva (Theron *et al*., 2002). Furthermore, gastric–oral transmission was also suggested after the organism was cultured from vomitus (Siu *et al*., 1999; Parsonnet *et al*., 1999). This was further strengthened by Perry *et al.,* who found that this transmission correlates with exposure to an infected family member with gastroenteritis, especially with vomiting (de la Luz Sanchez *et al*., 2006). Other possible ways of transmission include water-borne transmission (Graham *et al*., 1991) and vector-borne transmission (Hoffman *et al*., 1997; Grubel *et al*., 1998). Environmental or animal reservoirs were investigated as sources of *H. pylori* infection. Food, animals, and water sources have been suggested as reservoirs outside the human gastrointestinal tract, and *H. pylori* or its DNA was detected in each of these sources (Kurosawa *et al*., 2000). Therefore, it is evident that the current knowledge on the exact modes of transmission is not optimal, and further studies are needed to clarify this clinically and epidemiologically important aspect of *H. pylori*.

Pathogenesis of *H. pylori* infection

The primary disorder, which occurs after colonization with *H. pylori*, is chronic active gastritis. This condition can be observed in all *H. pylori*-positive subjects. The intragastric distribution and severity of this chronic inflammatory process depend on a variety of factors, such as characteristics of the colonizing strain, host genetics and immune response, diet, and the level of acid production. *H. pylori*-induced ulcer disease, gastric cancer, and lymphoma are all complications of this chronic inflammation; ulcer disease and gastric cancer in particular occur in those individuals and at those sites with the most severe inflammation. Understanding of these factors is thus crucial for the recognition of the role of *H. pylori* in the etiology of upper gastrointestinal pathology.

The gastric mucosa is well protected against bacterial infections. *H. pylori* is highly adapted to this ecologic niche, with a unique array of features that permit entry into the mucus, swimming and spatial orientation in the mucus, attachment to epithelial cells, evasion of the immune response, and, as a result, persistent colonization and transmission. To colonize the stomach, *H. pylori* must survive the acidic pH of the lumen and use its flagella to burrow into the mucus to reach its niche, close to the stomach's epithelial cell layer (Amieva & El-Omar, 2008). Many bacteria can be found deep in the mucus, which is continuously secreted by mucus-secreting cells and removed on the luminal side. To avoid being carried into the lumen, *H. pylori* senses the pH gradient within the mucus layer by chemotaxis and swims away from the acidic contents of the lumen towards the more neutral pH environment of the epithelial cell surface (Schreiber *et al*., 2004).

Figure 2.4: Pathogenesis of *H. pylori* infection



To survive in the presence of acid produced in the stomach *H. pylori* has to counteract this acidic environment. *H. pylori* produce an important enzyme, urease, which hydrolyses urea into NH_3 and CO_2. This enzyme has an essential role in the *H. pylori* infection as observed in urease-defective bacteria mutants which cannot colonize the stomach (Montecucco and Rappuoli, 2001). Urease causes damage to the epithelium through the production of ammonia, that in conjunction with neutrophil metabolites (Megraud *et al*., 1992), form carcinogenic agents that might participate in the development of gastric malignances ((Megraud *et al*., 1992), Suzuki *et al*., 1992). Ammonia is capable of causing different cell alterations, including swelling of intracellular acidic compartments, alterations of vesicular membrane transport, repression of protein synthesis and ATP production, and cell-cycle arrest (Montecucco and Rappuoli, 2001). Urease might also help to the recruitment of neutrophils and monocytes in the mucosa and to the production of pro-inflammatory cytokines (Harris *et al*., 1996).

H. pylori LPS are essential components of the bacterial outer membrane that induces a low immunological response in contrary to other bacteria (Moran, 1995). However, despite the low immunological activity of LPS, *H. pylori* colonization is associated with an inflammatory response, because *H. pylori* has the ability of activate mononuclear cells by LPS-independent mechanisms as well as other bacterial surface molecules (Moran, 1998). Another important component of the cell wall of *H. pylori*, peptidoglycan, interacts with the intracellular pattern recognition receptor Nod1 (an intracellular sensor for peptidoglycan from Gram-negative bacteria), this interaction leads to activation of NF-κB signaling, with the subsequent secretion of the inflammatory molecules IL-8 (Viala *et al*., 2004) and β-defensin-2 (Boughan *et al*., 2006). On the other hand, peptidoglycan translocation of *H. pylori*, leads to enhanced PI3K-AKT signaling, which mediates protection from apoptosis and cell migration, both phenotypes related to carcinogenesis (Nagy *et al*., 2009).

Approximately 4% of the *H. pylori* genome is predicted to encode outer membrane proteins (OMPs) many of which serve as adhesins (Wroblewski *et al*., 2010), epithelial adhesion provides to *H. pylori* better access to epithelial surface and in this way contributes to deliver bacterial toxins such as CagA (Yamaoka, 2010). Fucosylated ABO blood group antigens and sialyl-Lewis^x and sialyl-Lewis^a antigens (sLe^x and sLe^a) have been identified as functional receptors for *H. pylori* (Boren *et al*., 1993).

The outer membrane inflammatory protein (OipA), identified in 2000 (Yamaoka *et al*., 2000), is involved in the attachment of *H. pylori* to gastric epithelial cells in vitro (Yamaoka, 2010). OipA is also involved in up-regulation of matrix metalloproteinase 1 (MMP-1), in inhibition of glycogen synthase kinase 3β (GSK-3β) and in β-catenin translocation to the nucleus, this protein also induces an inflammatory response and actin rearrangement through phosphorylation signaling pathways (Wroblewski *et al*., 2010), although, most of these are also involved in cag-PAI signaling alterations, suggesting that there might be some interaction between OipA and cag-PAI, it is important to note that it has been found a correlation coefficient of 0.82 between the oipA and cagA positivity in some virulent strains (Yamaoka *et al*., 2002).

Blood group antigen binding adhesin (BabA) has been thought to be the primary protein involved in adherence to the gastric mucosa (Boren *et al*., 1993). It is encoded by the babA2 gene (the babA1 although is present, is silent because of lack of an initiation codon (Mobley *et al*., 2001) and binds to fucosylated Lewis^{b} antigen. Some studies report that the presence of babA2 is associated with duodenal ulcer and gastric cancer and found in conjunction with cagA and vacA s1 allele, provides a greater risk of develop a severe gastric disease (Gerhard *et al*., 1999).

Sialic acid-binding adhesin (SabA) is responsible of *H. pylori* binding to syalylated Le^{x} and Le^{a} in epithelial cells. Infiltration of neutrophils into the gastric mucosa is a characteristic feature of chronic gastritis caused by *H. pylori* and SabA binds to neutrophils through these sialylated carbohydrates and induces oxidative burst in these cells, which has a consequence of produce oxidative damage in gastric epithelium. It has been proposed that SabA-positive status is associated with gastric cancer, intestinal metaplasia, and corpus atrophy and negatively associated with duodenal ulcer and neutrophil infiltration (Yamaoka, 2008).

VacA is a pore-forming cytotoxin identified in supernatants of *H. pylori* broth cultures that cause aberrant vacuolation of cultured cells. This cytotoxin is secreted from the bacteria as a large 140-kilodalton polypeptide and latter trimmed at both ends to finally deliver it in an active form to host cells, where it exerts its activity.

VacA induces multiple cellular activities, the best studied is the alteration on endosomal maturation which consequently leads to vacuolation of epithelial cells, VacA is also capable of induce membrane-channel formation, cytochrome c release from mitochondria, and binding to cell-membrane receptors activating a proinflammatory response (Amieva & El-

Omar, 2008). VacA has the ability to cause leakage of ions and small molecules, by disrupting the barrier function of tight junctions and also inhibit T-cell activation and proliferation (Gebert *et al*., 2003).

All strains of *H. pylori* contain the vacA gene, although, there is considerable genetic diversity among them, and consequently the cytotoxicity activity of the toxin varies between strains (Amieva & El-Omar, 2008). The diversity observed is attributed to variations in vacA gene structure within the signal (s) region, the middle (m) region, and the intermediate (i) region (Wroblewski *et al*., 2010).

The amino terminus contains the signal sequence that shows allelic variability and has been classified into different types. Strains with s1 allele secrete an active toxin and are also highly associated with ulcers and gastric cancer (Atherton *et al*., 1995), however, s1/s2 combination or s2 genotypes are found in patients with gastric cancer (Lopez-Vidal *et al*., 2008). The middle region of the gene also shows allelic variation, with m1 subtype having stronger vacuolating activity and it is associated with an increased risk for development of gastric epithelial injury and gastric cancer (Yamaoka, 2010). The clinical isolates of *H. pylori* have been grouped into two broad families defined as type I and type II on the basis of whether they have a functional pathogenicity island (cag-PAI), and secrete an active vacuolating cytotoxin (VacA). Type I strains are positive for all these characteristics. In contrast, type II strains do not have a functional cag-PAI and possess the vacA gene that encodes for non-toxic protein (Censini *et al*., 1996, Tomb *et al*., 1997).

Colonization of the human stomach by *H. pylori* virulent strains is associated with a significantly increased risk for the development of several gastric diseases, such as gastric cancer. These highly virulent *H. pylori* strains harbor a (cytotoxin-associated genes) pathogenicity island (cag-PAI) that encode proteins that are components of a type IV secretion system (T4SS) apparatus and the CagA effector protein into host target cells (Castillo-Rojas *et al*., 2004). T4SS translocates the bacterial oncoprotein CagA and peptidoglycan that induces pro-inflammatory chemokine and cytokine secretion, including interleukin-8 (IL-8) in gastric epithelial cells (Figueiredo *et al*., 2005). The hallmark of *H. pylori* virulent strains is the presence of cag-PAI that induce of gastric mucosal inflammation, which is a risk factor for developing severe gastric pathologies (Antonio-Rincon *et al*., 2011).

The presence of flanking direct repeated sequences 605 (IS605) in the cag-PAI have been reported to cause rearrangements and partial or total deletions of it. This has generated *H.*

pylori strains with varying virulence, and consequently, the clinical outcome of the infection (Censini *et al*., 1996). A systematic mutagenesis study with isogenic mutants in each of the cag-PAI genes, reported that 14 genes out of 27 of cag-PAI are essential for CagA translocation and induction of IL-8 secretion in gastric epithelial cells (Aguilar *et al*., 2001).

CagA protein is the only known effector protein translocated to the host cell by the T4SS (Backert *et al*., 2010). It was firstly described as a very immunogenic protein in humans infected with cytotoxin-producing strains (Covacci *et al*., 1993). After that, its association with gastric cancer and peptic ulcer was well established. Recent reports suggest that CagA is able to down regulate the vacuolation effects of VacA on host cells, and conversely, VacA is able to down regulate CagA activity (Oldani *et al*., 2009). Clinical isolates that contain the cag-PAI generally are babA, oipA and vacA s1 positive, Yamaoka proposes that these virulence factors may interact with each other with a certain biological significance; therefore, these factors interact synergistically with each other to induce gastric diseases (Yamaoka, 2010).

CagA is encoded by the cagA gene, located at one end of the cag-PAI (Censini *et al*., 1996). One copy per genome is present in most of the strains whose genome has been sequenced but strains with two gene copies might exist, as revealed by the genome sequence of the Amerindian strains *H. pylori* Shi470 and V225d, although functionality of both copies in these strains is unknown (Mane *et al*., 2010). It has been estimated that 60-95% of strains worldwide carry the cagA gene, which is different between geographic regions (Hatakeyama, 2004). However, the complete cag-PAI is important for the translocation of this protein and the completeness of the island may impact on the association of CagA presence and the outcome of the disease.

Other main feature of CagA protein is its high variable size, which range from 120-145 kDa (Covacci *et al*., 1993). This variation is due to the presence of polymorphism at the carboxy-terminal region given by the presence of repeat sequences called EPIYA motifs, which are present in a copy number varying from one to seven (Xia *et al*., 2009). Four major types of EPIYA motifs (A, B, C, and D) have been described based on the specific amino acid sequence that flanks the Glu-Pro-Ile-Tyr-Ala motif at both sides (Hatakeyama, 2004).

It has been proposed that EPIYA motifs of CagA act as ‘‘master keys’’ that can potentially be tyrosine-phosphorylated by host kinases and are capable to interact with a wide range of different host cell proteins (Backert *et al*., 2010). In the same way, the observation that the

EPIYA-region is unstructured and can adopt multiple conformations mimicking host substrates of kinase families, thus manipulating eukaryotic cellular biochemistry during infection, provides further support for this "master key" hypothesis (Nesic *et al*., 2010).

In contrast to the polymorphic C-terminal EPIYA region of CagA, N-terminal region is well conserved among CagA proteins (Hatakeyama, 2011). The N-terminal CagA region is required for the membrane association of CagA in polarized epithelial cells (Bagnoli *et al*., 2005) and is important for its translocation into host cells (Murata-Kamiya *et al*., 2010), the first 200 amino acids at the N-terminus of CagA form a membrane-binding domain in *H. pylori*, mediating proper translocation of the CagA protein via the T4SS (Steininger *et al*., 2011).

Clinical features

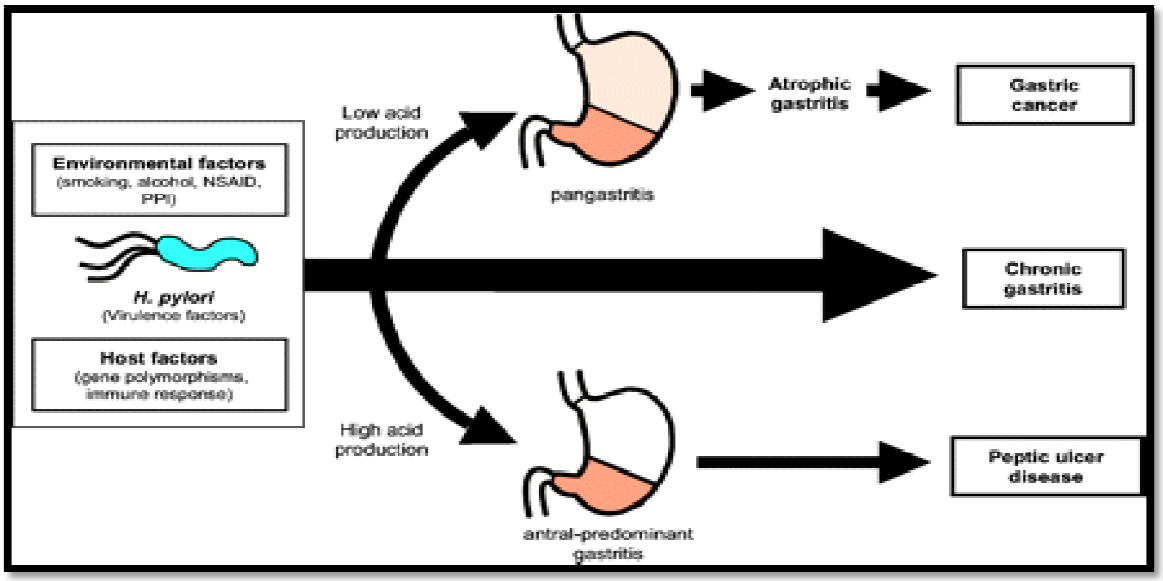
Chronic *H. pylori* –associated gastritis per se is asymptomatic but the initial acquisition of the infection cause acute gastritis with hypochlorhydria which may cause abdominal pain, nausea and vomiting that resolve within a few days (Fischer *et al*., 2001). Uncomplicated peptic ulcers typically cause epigastric pain and less commonly, nausea, vomiting and weight loss, whereas some ulcers (particularly NSAID ulcers) are asymptomatic. The classically described pain of duodenal ulcer is felt as a growing or burning sensation, often with a relation to meals; occurring 1-3 hours after meals and /or at night and relieved by food. Gastric ulcer pain is instead often precipitated by food. However symptoms are actually very poorly discriminatory for ulceration site and even for whether or not an ulcer is present. Examination usually reveals epigastric tenderness but may be normal.

H. pylori related pathological conditions

Disease types

Although gastric colonization with *H. pylori* induces histologic gastritis in all infected individuals, only a minority develop any apparent clinical signs of this colonization. It is estimated that *H. pylori*-positive patients have a 10 to 20% lifetime risk of developing ulcer disease and a 1 to 2% risk of developing distal gastric cancer (Ernst and Gold**,** 2000; kuipers *et al*., 1995). The risk of development of these disorders in the presence of *H. pylori* infection depends on a variety of bacterial, host, and environmental factors that mostly relate to the pattern and severity of gastritis (Figure 2.5).

Figure 2.5: Schematic representation of the factors contributing to gastric pathology and disease outcome in *H. pylori* infection.



Acute and chronic gastritis

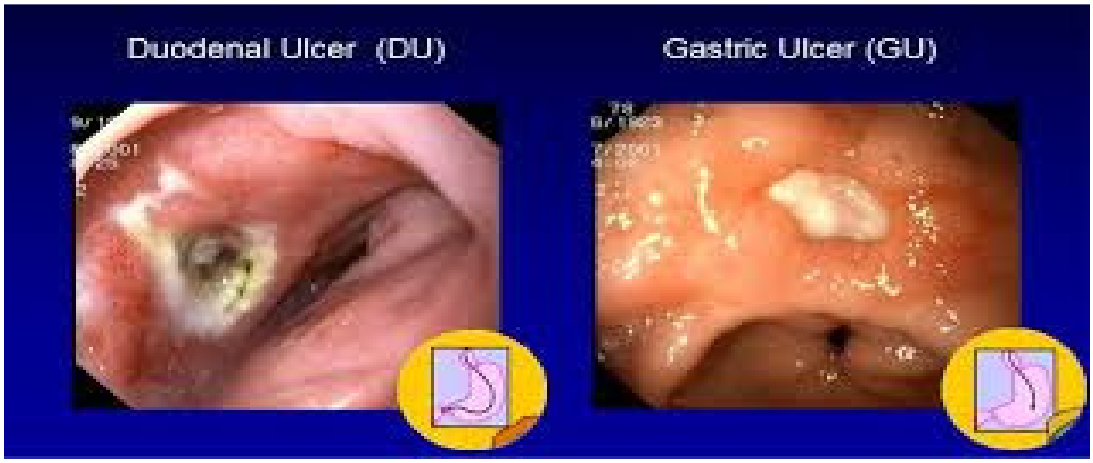
Many studies convincingly demonstrate that *H. pylori* colonization of the gastric mucosa is associated with gastritis, with chronic inflammatory cell infiltrate. The macroscopic nodular gastritis was significantly associated with active chronic gastritis and follicular gastritis. Colonization of the gastric antrum by *H. pylori* is graded as mild, moderate or marked (Uc and Chong, 2002).

Chronic *H. pylori* –associated gastritis per se is asymptomatic but the initial acquisition of the infection cause acute gastritis with hypochlorhydria which may cause abdominal pain, nausea and vomiting that resolve within a few days (Fischer *et al*, 2001).

Peptic ulcer disease (PUD)

Gastric or duodenal ulcers (commonly referred to as peptic ulcers) are defined as mucosal defects with a diameter of at least 0.5 cm penetrating through the muscularis mucosa.

Figure 2.6: Peptic ulcers



Gastric ulcers mostly occur along the lesser curvature of the stomach, in particular, at the transition from corpus to antrum mucosa (Veldhuyzen van Zanten *et al*., 1999). Both gastric and duodenal ulcer diseases are strongly related to *H. pylori* infection. In initial reports from all over the world in the first decade after the discovery of *H. pylori*, approximately 95% of duodenal ulcers and 85% of gastric ulcers occurred in the presence of *H. pylori* infection (Kuiper *et al*., 1995). Several cohort studies estimated that the lifetime risk for ulcer disease in *H. pylori*-positive subjects is 3 to 10 times higher than in *H. pylori*-negative subjects (Nomura *et al*., 1994) and that 10 to 15% of *H. pylori*-positive subjects developed ulcer disease during long-term follow-up (Sipponen *et al*., 1990).

Eradication of *H. pylori* dramatically changes the natural course of ulcer disease and almost completely prevents ulcer recurrence (Hentschell *et al*., 1993**).**

Non-ulcer dyspepsia

Non-ulcer or functional dyspepsia is defined as the presence of symptoms of upper gastrointestinal distress without any identifiable structural abnormality during diagnostic work-up, in particular including upper gastrointestinal endoscopy. Thirty (30%) to 60% of patients with functional dyspepsia carry *H. pylori*, but this prevalence is not much different from that in the unaffected population (Talley and Hunt, 1997).

Atrophic gastritis, intestinal metaplasia, and gastric cancer

Chronic *H. pylori*-induced inflammation can eventually lead to loss of the normal gastric mucosal architecture, with destruction of gastric glands and replacement by fibrosis and intestinal-type epithelium. This process of atrophic gastritis and intestinal metaplasia occurs in approximately half of the *H. pylori*-colonized population, first in those subjects and at those sites where inflammation is most severe (Kuipers *et al*., 1995). The risk for atrophic gastritis depends on the distribution and pattern of chronic active inflammation. As such, subjects with decreased acid output show a more rapid progression towards atrophy (kuipers *et al*., 1996). Areas of gland loss and intestinal metaplasia extend with time multifocally, and although they do not give rise to any specific symptoms, they increase the risk for gastric cancer by 5- to 90-fold depending on the extent and severity of atrophy (Sipponen *et al*., 1985).

Evidence that *H. pylori* increases the risk of gastric cancer development via the sequence of atrophy and metaplasia originates from various studies, in which it was shown that *H. pylori*-positive subjects develop these conditions more often than do uninfected controls (Kuipers, 1998). This is supported by data that showed geographical associations between the prevalence of *H. pylori* and the incidence of gastric cancer (Forman *et al*., 1990, The Eurogast Study Group, 1993**).** The risk of development of atrophy and cancer in the presence of *H. pylori* is again related to host and bacterial factors, which influence the severity of the chronic inflammatory response. As such, the risk is increased in subjects colonized with *cagA*-positive strains (kuipers *et al*., 1995**,** Parsonnet *et al*., 1997), but also in those with a genetic predisposition to higher IL-1 production in response to colonization (El-Omar *et al*., 2000**).**

Gastric MALT lymphoma

The association of *H. pylori* and MALToma is an established fact. The gastric mucosa does not normally contain lymphoid tissue, but MALT nearly always appears in response to colonization with *H. pylori*. In rare cases, a monoclonal population of B cells may arise from this tissue and slowly proliferate to form a MALT lymphoma. Nearly all MALT lymphoma patients are *H. pylori* positive (Eidt *et al*., 1994), and *H. pylori*-positive subjects have a significantly increased risk for the development of gastric MALT lymphoma (Personnet *et al*., 1994). Because of the diagnostic controversies and the relative rarity of this disorder, the

exact incidence in *H. pylori*-positive subjects is unknown, but MALT lymphomas occur in less than 1% of *H. pylori*-positive subjects **(**Parsonnet and Isaacson, 2004**).**

Gastro-esophageal reflux disease (GERD)

Yet another highly controversial topic is the role of *H. pylori* in the pathogenesis of gastro-esophageal reflux disease (GERD). Some studies suggest that *H. pylori* protects human subjects from developing GERD (Blasher, 1998), whereas others postulate a causative association between them. Some studies have identified an association between the CagA-positive strains and the increased acid secretion that in turn leads to gastro-esophageal reflux (Fallone *et al*., 2000**).** The causative association between *H. pylori* and GERD needs further research for confirmation.

Extragastroduodenal disorders

H. pylori has been linked to a variety of extragastric disorders. These include coronary heart disease, dermatological disorders such as rosacea and idiopathic urticaria, autoimmune thyroid disease and thrombocytopenic purpura, iron deficiency anemia, Raynaud's phenomenon, scleroderma, migraine, and Guillain-Barré syndrome. The underlying hypothetical mechanisms include chronic low-grade activation of the coagulation cascade, accelerating atherosclerosis, and antigenic mimicry between *H. pylori* and host epitopes leading to autoimmune disorders **(**Gasbarrini *et al*., 2004**)**.

Growth faltering in children

Several studies have shown that *H. pylori* infection in childhood is associated with growth faltering (Goggin *et al*., 1997; Correa *et al*., 2006). However, these studies are confounded by the coexistence of variables such as poor socioeconomic status, which may contribute to both the development of malnutrition and the early *H. pylori* colonization. Therefore, *H. pylori* and growth faltering may be mere associations rather than cause and effect.

Diagnosis of *H. pylori* infection

Testing for *H. pylori* infection has become a very important part of the diagnostic process for gastric and duodenal inflammatory disease, since the presence or absence of infection determines the type of treatment to be applied. Testing is also a useful means of monitoring the effectiveness of courses of antimicrobial treatment. Various tests have been developed for the detection of *H. pylori*, each with their specific advantages and disadvantages (Table 2.4).

Table 2.4: Diagnostic methods for detection of *H. pylori* infection

Diagnostic method	Sensitivity and specificity[a]	Typical application	Remarks
Invasive methods			
Histology	>95%	"Gold standard" in routine hospital diagnostics	Requires expert pathologist; also provides histological data on inflammation and atrophy
Culture biopsy	>95%	Alternative gold standard	Allows for testing of antimicrobial sensitivity; requires specific microbiological expertise
Rapid urease (CLO) test	>90%	Cost-effective and rapid test	Requires an additional test for confirmation of *H. pylori* infection
Noninvasive methods			
Urea breath test	>95%	Alternative gold standard	Very useful, reliable test to evaluate success of eradication treatment of *H. pylori*; limited availability due to requirement of expensive equipment
Fecal antigen test	>90%	Not widely used yet	Simple test and may be reliable for evaluation of success of eradication treatment of *H. pylori*
Serology	80-90%	Mainly used for epidemiological studies	Insufficient reliability for routine screening; cannot prove ongoing infection due to immunological memory

↲*a* Global range, depending on regional variations and subjects.

The available tests are generally divided into invasive tests, based on gastric specimens for histology, culture, or other methods, and noninvasive tests, based on peripheral samples, such

as blood, breath samples, stools, urine, or saliva for detection of antibodies, bacterial antigens, or urease activity.

Generally, biopsy cannot be justified unless one wishes to isolate the organism for antibiotic sensitivity testing or there is a clear clinical indication for UGIE. If one opted to test for *H. pylori* by biopsies with UGIE, it needs a specimen from multiple regions of the stomach, including antrum, body and transitional zones (i.e., cardia and incisura).

Several factors, including the need for endoscopy, pretest probability of infection, local availability, and an understanding of the performance characteristics of the individual tests, influence choice of evaluation for an individual patient.

Invasive methods

The collection of biopsy specimens from inflamed or ulcerated regions of the stomach and duodenum during invasive endoscopy is considered to be the reference method for diagnosing *H. pylori* infection. The biopsy material can be examined using one, or more, of three different test 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 endoscopy. 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 seven days.

Culture is generally regarded as the 'gold standard' for detecting a bacterium. For H pylori, however, the success of the technique depends on local technique and 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 (DeCross & Puera, 1992; Perez-Perez *et al*., 1988).

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-Hopps stains has been shown to be 100%, compared with H&E (Madan *et al*., 1988), 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.

Rapid urease test (RUT)

The ability of the organism to split urea using the enzyme urease is used to identify *H. pylori* in tissue biopsy specimens. A specimen is placed on a commercially available urea-containing medium. The hydrolysis of the urea by urease brings about a color change in the pH-sensitive chemicals in the medium allowing the investigator to identify the presence of the organism. The accuracy of the test is dependent on the number of tissue specimens tested, the location from which the biopsy is obtained, the bacterial load and previous use of antibiotics and proton pump inhibitors as well as the prevalence of the infection in the community (Bourke and Drumm, 2004).

In infected tissues, the change occurs within about one hour, so that the results of the RUT test are often available while the patient is still at the endoscopy 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.

Non-invasive methods

Several techniques for non-invasive diagnostic tests for *H. pylori* have been developed and tests based on three of these are in widespread use.

Urea breath test (UBT)

Urea breath test (UBT) is a simple, non-invasive test based on *H. pylori* urease, and a specific indicator of *H. pylori* infection (Atherton & Spiller, 1994). The procedure requires a patient to attend a centre fasting, to eat a standard meal and then ingest urea labelled with C-14 or C-13. The presence of large amounts of *H. pylori* urease results in the production of labelled carbon dioxide, which is absorbed into the blood and excreted in the breath. Samples of breath are collected before and sometime after the ingestion of labelled urea and the amount of labelled 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 centres, 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 has ended, should that be necessary.

Serology

People infected with *H. pylori* generally have specific IgG and IgA antibodies circulating in their blood and these can be detected by serological tests. Tests for the detection of antibodies to *H. pylori* circulating in blood, or found in saliva, have excellent sensitivity and specificity of above 95% and are cheap and simple compared with invasive techniques (DeCross & Puera, 1992). 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 past (NIH Consensus Conference, 1994). Because there are different strains of H pylori, antigen for antibody manufacture 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. 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-IgG methods tend to be favoured (Perez-Perez *et al*., 1988). The commercially available assays are of two sorts: either microtitre-plate assays for use in a laboratory, or near-patient testing devices. Both types of assay 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 and near-patient testing devices perform equally well compared with standard techniques.

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.

At least one commentator has suggested that for *H. pylori* infection, serology testing may be the "gold standard" (Blaser, 1990). Essentially, every person whose gastric biopsy can be shown to harbour *H. pylori* has evidence of a systemic humoral 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 histologic 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.

Stool Antigen Test

Recently, new tests for *H. pylori* infection have been developed that rely on the detection of specific antigens in the stools of infected individuals. The *Helicobacter pylori* Stool Antigen test (HpSA) is an enzyme immunoassay. The initially developed stool antigen test was a polyclonal antibody test (Premier Platinum HpSA test; Meridian Diagnostic Inc., Cincinnati, OH, USA) and was found to have variable sensitivities and specificities for the diagnosis of *H. pylori* infection. The development of a new ELISA test using monoclonal antibodies (Femtolab *H. pylori*; Connex, Martinsried, Germany) gave a new dimension and greater precision for stool antigen testing. In comparative studies, stool antigen test using monoclonal antibodies showed a higher sensitivity than the polyclonal test (This *et al*., 2000). This test has demonstrated sensitivities, specificities and positive and negative predictive values of 98%, 99%, 98% and 99%, respectively (Konstantopopulos *et al*., 2003) proving that monoclonal stool antigen enzyme immune assay is an excellent tool in diagnosing *H. pylori* infection and confirming eradication.

H. pylori antigen detection in stool is a rapid, non invasive, easy to perform test that can be used to detect active infection, monitor effectiveness during therapy and to confirm cure after antibiotic use (Vaira *et al*., 2002). The ease to collect the specimen, especially in children, in whom endoscopy would be difficult and the non requirement of specially trained staff to collect and perform the test adds to the tests advantage. Also, prior preparation of the patient is not necessary unlike in upper gastrointestinal endoscopy. The sensitivity and specificity of this test to the other detection methods of *H. pylori* have been confirmed and documented (Vaira *et al*., 2000). HpSA has a 95% correlation with reference methods such as endoscopy, histology and urea breath test. It is more developed as alternative to the urea breath test (Gisbert *et al*., 2006).

MATERIALS AND METHODS

The present study was carried out on 92 consecutive adult patients attending the Department of Gastrointestinal, Hepatobiliary and Pancreatic Diseases (GHPD) of BIRDEM General Hospital for diagnostic endoscopy.

Written informed consent was obtained from all cases. The research study was approved by the ethics committee of BRAC University.

Place of the study:

All laboratory works were carried out in the Ibrahim Medical College, BIRDEM, Dhaka.

Study Period:

The study was conducted during the period of July' 2012 to February' 2014.

Study Design:

This was a cross sectional study.

Study population:

Inclusion criteria:

i. Patients having gastrointestinal complaints, mainly dyspeptic symptoms (as nausea, vomiting and epigastric pain) will be included.

Exclusion criteria:

i. Patients with hepatic, pulmonary, renal and cardiac diseases,
ii. Patients who undergone partial or complete gastrectomy,
iii. Those with a prior *H. pylori* eradication therapy,
iv. Those who were treated with any antibiotics, colloidal bismuth compounds, proton pump inhibitors (PPI), or H_2 blocker within the last four weeks.

Control:

Another 31 infants aged 1 day to 30 days was enrolled from SCABU (Special Care Baby Unit) of BIRDEM General Hospital-2 as disease control. Stool sample was collected from those infants to detect *H. pylori* antigen (Ag).

Collection of samples:

Gastric biopsy

While diagnostic upper gastrointerstinal endoscpic examination was done, the endoscopic findings were recorded and one piece of gastric biopsy was taken for rapid urease test (RUT).

Blood

For detection of *H. pylori* antibodies 2.5 ml blood was collected from the patients. After collection, blood was kept at room temperature for at least half an hour followed by centrifugation at 1500 rpm for 10 minutes. The serum was aliquoted into microcentrifuge tube and stored at –20°C. Later on the serum was used for detection of anti *H. pylori* antibodies.

Stool

Stool sample was collected from each patient and control in a clean, dry, waterproof container containing no detergent, preservatives or transport media**.** After collection stool samples were examined for *H. pylori* antigen within 6 hours. If test not done immediately, sample was stored at -20^{o}C.

Laboratory procedures:

I. Rapid Urease Test (RUT) of biopsy specimen:

Immediately after collection, biopsy specimen was suspended in 80% urea medium. Then the medium was incubated at 37^{0}C and examined after 4 hours or after over-night incubation to detect urease activity. Urease positive isolates changed the colour of the medium from yellow to pink (Owen, 1996).

II. Stool antigen detection:

a. Processing of stool:

- For solid specimen, the cap of the specimen collection tube was unscrewed and then specimen collection applicator will be stabbed randomly into fecal specimen in at least 3 different sites to collect approximately 50 mg of feces.
- For liquid specimen, the dropper was held vertically, the specimen was aspirated and then transferred 2 drops (approx 80 µL) into specimen collection tube containing extraction buffer.

Then the cap was tightened, collection tube was shaken vigorously using vortex mixer and then centrifuged for 5 minutes at 4000rpm.

b. Stool antigen assay (HpSA)

Diluted stool samples were analyzed using the ABON One step *H. pylori* Antigen test device that is a lateral flow chromatographic immunoassay for detection of *H. pylori* antigen.

- The specimen collection tube was held upright
- Tip of the collection tube was broken off and inverted to transfer 2 drops of extracted specimen to specimen wells of test device.
- Then timer was started.
- The result was read at 10 minutes after dispensing specimen.
- Appearance of two distinct lines indicated positive result and one line in the control region indicated negative. Disappearance of control line indicated invalid result.

III. Serum antibody detection by Enzyme-Linked Immunosorbent Assay (ELISA):

Serum samples were tested for the presence of anti *H. pylori* IgG and IgA antibodies. Test was performed by using DRG *Helicobacter pylori* IgG and IgA ELISA kit according to manufacturer instruction.

Statistical analysis

Culture is usually considered as gold standard to detect *H. pylori* infection. But culture was not performed in our study because of our limitations. RUT and serology were done and used to determine disease positive and negative cases. In serology, anti-*H. pylori* IgG and IgA antibodies were examined. As exposure to *H. pylori* occurs at a very early stage of age (1-5 yrs), almost all patients became positive for IgG antibody in this study. So both RUT and IgA positive cases were taken as “disease positive”. RUT & IgA along with stool Ag positive cases were “True positive”. Both RUT & IgA positive but stool Ag negative cases were “False negative”. Alternatively, thirty one infants aged between 1 to 30 days from neonatal ICU were taken as “disease negative” cases. It was predicted that those infants were not

exposed to *H. pylori*. Among them, 30 infants were stool Ag test negative. These are "True negative" cases. One infant found positive which indicated "False positive". On this basis, statistical analysis was done in the study.

RESULTS

The present study was carried out on 92 patients suffering from dyspepsia and other upper gastro intestinal tract symptoms as nausea, vomiting, epigastric pain. From these patients gastric biopsy specimens were taken for detection of *Helicobacter pylori* infection by rapid urease test (RUT). In addition, stool samples were taken for the detection of *H. pylori* antigen and blood for detection of anti-*H. pylori* IgA and IgG antibodies.

Simultaneously, stool samples were examined as disease control from another 31 infants aged from 1 day to 30 days.

Table 4.1: Results of Rapid Urease Test (RUT) and *H. pylori* stool antigen (Ag) detection in ulcer and erosion patients (n=92).

Endoscopic diagnosis	Number of cases	Number of positivity by n (%)	
		RUT	Stool Ag
Ulcer	19	10 (52.63%)	14 (73.68%)
Erosion	73	30 (41.09%)	44 (60.27%)
Total	92	40 (43.48%)	58 (63.04%)

Table 4.1 shows the rate of positivity of RUT and *H. pylori* stool antigens (Ag) test in ulcer and erosion patients. Among 92 adult patients undergone endoscopic examination, 19 were found having ulcer and 73 having erosion. Among 19 ulcer cases, 10 (52.63%) cases were RUT positive and 14 (73.68%) cases were stool antigen positive. Out of 73 erosion cases, 30 (41.09%) cases were positive by RUT and 44 (60.27%) cases were positive by Stool antigen test. Ulcer cases showed higher positivity in antigen detection and RUT than erosion cases. Among total 92 adult patients, total 40 (43.48%) cases were RUT positive and 58 (63.04%) cases were stool antigen positive.

Table 4.2: Relation of Rapid Urease Test (RUT) with *H. pylori* stool antigen (Ag) detection in ulcer and erosion patients (n= 92).

Test	No. of cases	Stool Ag positive	Stool Ag negative
RUT positive	40	39 (97.5%)	1 (2.5%)
RUT negative	52	19 (36.53%)	33 (63.46%)

H. pylori stool antigen (Ag) detection test was compared with RUT in **Table 4.2**. Among 40 RUT positive cases, 39 (97.5%) cases were stool antigen positive. Only 19 (36.53%) cases were stool antigen positive out of 52 RUT negative cases.

Table 4.3: Positivity of anti-*H.pylori* serum IgA and IgG antibodies in ulcer and erosion patients (n=92).

Endoscopic diagnosis	No of cases	Number of positivity by n (%)		
		Serum IgG	Serum IgA	Both Serum IgG & IgA
Ulcer	19	19 (100%)	12 (63.16%)	12 (63.16%)
Erosion	73	70 (95.89%)	46 (63.01%)	45(61.64%)
Total	92	89 (96.74%)	58 (63.04%)	57 (61.96%)

Serum from 92 adult patients having ulcer and erosion were examined for anti- *H. pylori* IgA and IgG antibodies and shown in **Table 4.3.** All 19 (100%) ulcer cases were positive for anti *H. pylori* IgG and 12 (63.16%) for IgA. Total 12 (63.16%) cases were positive for both IgG and IgA out of 19 ulcer cases. On the other hand, among 73 erosion cases 70(95.89%) cases were positive for anti *H. pylori* IgG and 46 (63.01%) cases for IgA. Total 45 (61.64%) erosion cases were positive for both IgG and IgA. Among 92 cases, IgA antibody was positive in 58 (63.04%) cases and IgG antibody was in 89 (96.74%) cases and both antibodies were present in 57 (61.96%) cases.

Table 4.4: Relation of Rapid Urease Test (RUT) with serology in ulcer and erosion patients (n= 92).

Test	No. of cases	Number of positivity by n (%)		
		Serum IgG	Serum IgA	Both IgA and IgG
RUT positive	40	39 (97.5%)	26 (65%)	25(62.5%)
RUT negative	52	50 (96.15%)	32 (61.54%)	31(59.62%)

Relationship between RUT and serology is showed in **Table 4.4.** Out of 40 RUT positive cases, 39 (97.5%) cases were IgG positive and 26 (65%) cases were IgA positive. Among 52 RUT negative cases, 50 (96.16%) were positive for IgG and 32 (61.54%) were positive for IgA. Both antibodies were present in 25 (62.5%) cases among 40 RUT positive cases and 31 (59.62%) cases among 52 RUT negative cases.

Table 4.5: Comparison of stool antigen (Ag) detection with serum anti *H. pylori* IgA and IgG in ulcer and erosion patients (n= 92).

Test	No. of cases	Number of positivity by n (%)		
		Serum IgG	Serum IgA	Both serum IgG & IgA
Stool Ag positive	58	57 (98.27%)	39 (67.24%)	38 (65.52%)
Stool Ag negative	34	32 (94.11%)	19 (55.88%)	19 (55.88%)

Comparison of stool antigen detection test with serology **(Table 4.5)** reveals, among 58 stool antigen positive cases, 57 (98.27%) were positive for IgG and 39 (67.24%) were for IgA. On the other hand, 32 (94.11%) were positive for IgG and 19 (55.88%) were positive for IgA out of 34 stool antigen negative cases. Among 58 stool antigen positive cases, 38 (65.52%) cases were positive for both IgA and IgG antibodies and 19 (55.88%) cases were positive for both antibodies out of 34 negative cases.

Table 4.6: Mean antibody titre (IgA and IgG) in stool antigen (Ag) positive and negative cases.

Stool Ag	No. of cases	Mean titre ± SD	
		Serum IgG	Serum IgA
Positive	58	2.09 ± 0.46	1.40 ± 1.01
Negative	34	1.94 ± 0.52	1.23 ± 0.95

Mean antibody titre was measured in stool antigen (Ag) positive and negative cases and showed in **Table 4.6.** In 58 stool Ag positive cases, IgG mean titre was (2.09 ± 0.46) and IgA mean titre was (1.40 ± 1.01). IgG mean titre was 1.94 ± 0.52 and IgA mean titre was 1.23 ± 0.95 in 34 stool Ag negative cases.

Table 4.7: Positivity of *H. pylori* stool antigen in 31 neonates taken as control.

Stool Ag detection	Result of *H. pylori* stool Ag	
	No	%
Positive	1	3.23
Negative	30	96.77

H. pylori stool antigen positivity of 31 infants as control is showed in **Table 4.7.** Among 31 infant stool samples, 1 (3.23%) was positive for stool antigen and 30 (96.77%) were negative for stool antigen.

Table 4.8: Performance of the *H. pylori* stool antigen assay (HpSA) method.

Test	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Diagnostic accuracy (%)
HpSA	100	96.77	96.30	100	98.25

PPV = Positive predictive value.

NPV = Negative predictive value.

Performance of the (HpSA) method for *H. pylori* stool antigen detection **(Table 4.8)** in shows that the HpSA method for antigen detection had a sensitivity of 100% and specificity of 96.77%. Positive and negative predictive values of the HpSA method were 96.30% and 100% respectively. The diagnostic accuracy of the test was 98.25%.

DISCUSSION

Helicobacter pylori (*H. pylori*) is a major causative agent of several gastro-duodenal pathologies like chronic gastritis and peptic ulcer disease (Rauws and Tytgat, 1990). Furthermore, *H. pylori* plays a pivotal role in the pathogenesis of gastric cancer and gastric mucosa-associated lymphoid tissue (MALT) lymphoma (Asghar and Parsonnet, 2001). The severity of the inflammation is likely to underlie *H. pylori*-related diseases (Fock and Ang, 2010).

As the eradication of *H. pylori* has been shown to improve the outcome of peptic ulcer disease in terms of recurrence and complications, the accurate diagnosis of *H. pylori* infection is essential for the effective treatment and management of infection caused by *H. pylori*.

Numerous invasive and noninvasive diagnostic tests have been developed. Each of the techniques has advantages as well as disadvantages which will make it more or less appropriate depending on the clinical situation. It is now clear that the discussion over the different diagnostic methods cannot be oversimplified by reasoning only in term of which is the best diagnostic tool. (Chong, 2007; Iwanczak *et al*., 2005).

In the present study RUT, serology and *H. pylori* stool antigen (HpSA) test were used as diagnostic tools. The choice of diagnostic tests to determine *H. pylori* infection status depends on the sensitivity, specificity, reproducibility, availability, and rapidity of the results as well as the cost of the tests (Megraud, 1996). Unfortunately, none of the currently used methods can fulfill this criterion. One solution is to combine results of two or more techniques considered to be reliable, and compare with results of the method being evaluated.

Culture is usually considered as the gold standard for the diagnosis of any microorganism, but culture was not done in this study because of many drawbacks including that the microorganism is slow-growing, fastidious. Also it is time consuming and difficult to grow due to the usual non-abundance of bacteria and the strict conditions required for its growth like growth supplements and selective requirements (Hazell *et al.,* 1989; Heatly, 1995) .

Rapid urease test (RUT) of gastric biopsy material is the commonly used method to diagnose the presence of *H. pylori* by urease activity (Marshall *et al*., 1987; Hazell *et al*., 1987). In this study, Stool antigen (Ag) test (HpSA) was compared with serology and RUT.

In the present study after endoscopic examination of dyspeptic patients, 19 ulcer cases and 73 erosion cases were included. RUT was positive in 10 (52.63%) cases out of 19 ulcer cases. Among 73 erosion cases, 30 (41.09%) cases were found positive for RUT test **(Table 4.1)**. So it is clearly evident that ulcer cases were more RUT test positive than erosion cases. The overall RUT positive cases were 40 (43.48%). This finding is in harmony to the study done in Kuwait University, where 52% of the patients had a positive RUT test when they also used a single antral biopsy as we did (Siddique *et al.*, 2008). Akanda and Rahman (2011) also detected *H. pylori* by RUT in 56.4% case which is in agreement with our finding. However, our result was different from that of Refaay *et al*., (2006) who found 70% RUT positivity which is higher than our study. This may be due to the fact that in the RUT, false-negative results may occur because of irregular distribution of bacteria in the gastric mucosa (Chattopadhyay *et al*., 2005). So more than one biopsy specimen should be taken for getting more accurate result. But in the present study only a single biopsy was available which may influence the result.

In populations where *H. pylori* prevalence is high (≥ 20-25 percent), patients should be tested for *H. pylori* non-invasively, and given treatment to eradicate *H. pylori*. Regarding noninvasive techniques, the present study showed that stool antigen assay (HpSA) has produced promising results for the detection of *H. pylori* antigen in stool samples. This technique gave higher positivity in detecting *H. pylori* infection than RUT. *H. pylori* antigen was detected in 63.04% cases. Ulcer patients showed more positivity (73.68%) in antigen detection than erosion patients (60.27%) **(Table 4.1)**. The result was comparable to that of Nermin *et al.,* (2012) who stated that 66.7% of patients were found positive by testing their stool for *H. pylori* antigens. Refaay *et al*., (2006) found higher positivity (70%) in *H. pylori* stool antigen with a sensitivity of 83.3%. They also found that ulcer cases show more stool antigen positivity (80%) than erosion cases (41.7%) (Rafaay *et al*., 2006). From our study it is obvious that positivity by RUT and Stool Ag was higher in ulcer patients than erosion patients. So ulcer patients can be strongly suspected as *H. pylori* infection and be treated thereby. Simultaneously positivity of *H. pylori* infection should be carefully evaluated in patients having erosion in endoscopy for further evaluation. Regarding total samples, sensitivity of stool Ag was found to be higher than RUT both in ulcer and erosion cases.

In the present study, out of 40 RUT positive cases only one was stool Ag negative, rest 39 cases were found positive Stool Ag tests (HpSA). So it reveals that stool Ag test can efficiently detect *H. pylori* infection. Furthermore, among RUT negative cases 36.53% cases

were stool Ag positive **(Table 4.2).** It further emphasizes the higher sensitivity of Ag detection.

Pathological mechanism of *H. pylori* infection is associated with immunopathological response of host. When chronic gastritis occurs, a detectable specific humoral immunological response is established. The appearance of serum antibodies such as IgG and IgA may indicate an extensive immunoreactions caused by *H. pylori* infection.

In *H. pylori* infected patients, first IgM class antibodies develop. Then, both systematically and locally in gastric mucosa, IgG and IgA antibodies develop (Fox and Megraud, 2009). Sampling of gastric secretions from *H pylori*–infected individuals reveals a robust mucosal antibody response, primarily of the IgA isotype (Rathbone *et al*., 1986). Most patients infected with *H. pylori* produce a measurable systemic immune response, composed primarily of IgG (Perez-Perez *et al*., 1988). The levels of antibodies increase with age with the highest increases seen for IgG early during childhood and for IgA between childhood and adulthood. But circulating anti–*H. pylori* IgG antibodies persist at constant levels for years during infection and it′s titers also lasts for long periods of time after eradication of the infection. In contrast to serum IgG titers, IgA titers rise rapidly after infection and decrease if the infection is cleared. In addition, adults have about 15-fold higher IgA titers and 3-fold higher IgG titers than infants (Bhuiyan, 2010)

Serum IgA may be detected in variable ranges (39%-82%) of infected patients, and serum IgM is found rarely. These findings are consistent with a chronic infection usually acquired in early childhood. Assays to detect anti–*H. pylori* IgA in serum samples have variable sensitivities (39%-82%) compared with serum IgG immunoassays. Serum IgA studies may be useful in testing of symptomatic individuals with equivocal or negative IgG findings. In one study, more than 7% of patients with negative serum IgG results were found to have detectable anti–*H. pylori* serum IgA and symptoms consistent with *H. pylori* infection. Of 6 patients with IgA-positive, 5 had peptic ulcers documented by endoscopy (Jaskowski, 1997). Nystrom *et al.*, observed a direct relation between protection and IgA levels in the stomach (Nystrom *et al*., 2006). So, IgA positivity emphasizes the incidence of current infection.

In this thesis, serum anti- *H. pylori* IgA antibody was detected in 63.04% case and IgG antibody in 96.74% cases. Both antibodies were detected in 61.96% cases **(Table 4.3).** The percentage of serum IgG is very high because almost all the children get infected with *H. pylori* during their early childhood (Mahalanabis *et al*., 1996; Clemens *et al*., 1996). It is also

found that all IgA positive cases are also positive for IgG. So it clearly indicates current infection. Oluwasola *et al.*, (2012) also reported anti-*H. pylori* IgA positivity in 67.7% cases. Sharma *et al.,* (2012) also found 68.5% positivity by IgA serology. The anti-*H. pylori* IgA prevalence of our dyspeptic patients is similar to the values found in other studies for developing countries (Futagami *et al*., 1994; Kosunen *et al*., 2005). In the presence of IgG, IgA has been shown to correlate with active infection in 95% and 74% of cases of duodenal and gastric ulcers, respectively (Granberg *et al*., 1993). Yamamoto *et al.,* (1995) found a significant association between serum IgA titres and the development of atrophic gastritis. Li *et al.,* (2003), on the other hand found that in the absence of histological diagnosis of *H. pylori* in the gastric mucosa in chronic gastritis anti-*H. pylori* serum IgA is significantly higher in patients with severe intestinal metaplasia than in those with mild intestinal metaplasia. Increases in IgA titer against *H. pylori* antigens during active infections have been reported. IgA antibodies to *H. pylori* and low levels of pepsinogen I in patients' sera increase the risk of gastric carcinoma (Aromaa *et al*., 1996)

In the current study, almost all patients were found to be positive for IgG irrespective of RUT Stool Ag test status but IgA positivity was found to lower number of patients. It is obvious from our study, 62.5% case was positive for RUT & both antibodies. In addition, 65.52% case was positive for stool Ag & both antibodies. These were considered as true infection. Other than these, 59.62% RUT negative cases and 55.88% stool Ag negative cases were found positive for both antibodies. These cases should be carefully diagnosed by other methods or monitored for response after treatment **(Table 4.4 & 4.5)**.

The higher percentage of IgG positivity may be due to IgG is the predominant circulatory antibody than IgA and hence indicate that the predominant local production of IgA does not lead to high systemic IgA antibody levels in all patients. The same percentage of IgA and IgG seropositivity among RUT and stool Ag negative patients found may be due to the fact that IgG seropositivity may continue for many years, even proper antibiotics are used (Alim *et al*., (2010). Futagami *et al*., (1998) reported that the IgA response of gastric mucosa in chronic gastritis patients can be detected even in the quiescent period of negative *H. pylori* infection due to the recent exposure to the bacterial antigens.

It also appears in our study that sensitivity of IgA antibody and stool Ag test is almost equal which is much higher than RUT **(Table 4.1 & 4.3)**. This is supported by the result of Bhuiyan who observed that stool antigen test and IgA detect *H. pylori* infection almost at the same rate

and up to 90% of adults from Dhaka in Bangladesh were positive for *H. pylori*, assessed by stool antigen test and serologic anti- *H. pylori* IgA antibody. (Bhuiyan, 2010).

In our current study, IgA antibody mean titres were 1.40±1.01 and 1.23±0.95 in stool Ag positive and negative cases respectively. On the other hand, IgG antibody titre were 2.09±0.46 and 1.94±0.52 in case of Stool Ag positive and negative patients respectively **(Table 4.6).** There is no significant difference between IgA and IgG antibody titre among stool Ag positive and negative patients.

Culture is usually taken as gold standard to determine *H. pylori* infection. In the current study, culture was not carried out because of some limitations (e.g. it is slow growing, fastidious, require special medium & environment etc). Therefore, to determine specificity of the stool antigen test (HpSA), stool samples were collected from 31 neonates. These neonates were considered as 'Disese negative' cases because yet they were not exposed to *H. pylori.* Of 31 neonates stool samples, only one (3.23%) neonate was positive for *H. pylori* stool antigen test **(Table 4.7).** Fujimura *et al*., (2004) also found that stool antigen test was positive for 2% neonate with a positive PCR. Stray-Pedersen *et al*., (2007) detected *H. pylori* stool antigen in 15% neonates aged 7 days-1 month. These findings are similar to our study.

The sensitivity, specificity and diagnostic accuracy of HpSA test were 100%, 96.77% and 98.25% respectively **(Table 4.8).** The specificities of serum IgA were higher than serum IgG in relation to stool antigen test (73-98% for IgA and 47-77% for IgG) (Bhuiyan, 2010). A systematic review of the stool antigen test in untreated patients with *H. pylori* demonstrated overall sensitivity of 91%, specificity of 93%, and positive and negative predictive values of 92% and 87%, respectively (Gisbert and Pajares, 2004). A prospective multicentre European study evaluated the accuracy of the HpSA for active *H.pylori* infection in 501 patients, the results were compared to those obtained with invasive tests requiring endoscopy (RUT, H&E and the culture) and the non-invasive UBT. Several studies with HpSA in various regions of the world have shown comparable sensitivities (91-98%) and specificities (83-100%) (Kabir, 2003; Andrews *et al*., 2003; Gisbert and Pajares, 2001).

The noninvasive enzyme immunoassay HpSA is a quick and cost effective method. It does not require specialized expertise. The analytical technique of the immunoassay in stool samples can be performed easily in any laboratory. The test can be performed in conjunction with other diagnostic procedures. Feces can be obtained easily, even in new born children.

Spot samples of the stool are sufficient. Homogenization of the stool is not required. Previous studies have reported that a biopsy-based test has a lower detection rate for *H. pylori* in the presence of gastric atrophy (Yoo *et al*., 2007; Shin *et al*., 2009). The performance of a new stool antigen test on the other hand was comparable to that of other methods in the diagnosis of *H. pylori* infection for the screening population, even with the presence of atrophic gastritis/ intestinal metaplasia (Choi *et al*., 2011). Besides, it has been claimed that HpSA can be used for monitoring treatment success and that successful eradication of *H. pylori* can be confirmed after treatment (Arents *et al*., 2001). For abovementioned reasons, this method is more suitable for screening and epidemiological studies.

In conclusion, the study showed that highly sensitive and specific HpSA can be a reliable alternative to other techniques for diagnosing active *H. pylori* infection in non treated patients. It can be considered as a noninvasive first-line routine diagnostic test in our region. HpSA may be useful particularly in selection of the cases requiring endoscopic examination, in monitoring the response to treatment and in epidemiological studies.

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Appendices

Appendix 1

Patients's questionnaire form

Date:

Sample No:

1. **Patients's personal information:**

   Name: Age:

   Sex: Contact No:

2. **Patient type:**

   In Door: Out Door:

3. **Illness type and history:**
4. **Endoscopic finding:**

   Ulcer: Erosion:

5. **Sample collection:**

   Sample type: Biopsy, stool & Blood

Appendix 2

Calculation of Sensitivity, Specificity, PPV, NPV & Diagnostic accuracy:

$$\text{Sensitivity} = \frac{\text{True positive}}{\text{True positive + false negative}} \times 100$$

$$\text{Specificity} = \frac{\text{True negative}}{\text{False positive + true negative}} \times 100$$

$$\text{PPV} = \frac{\text{True positive}}{\text{True positive + false positive}} \times 100$$

$$\text{NPV} = \frac{\text{True negative}}{\text{True negative + false negative}} \times 100$$

$$\text{Diagnostic accuracy} = \frac{\text{True positive + True negative}}{\text{True positive + False positive + False negative + True negative}} \times 100$$

Appendix 3

Rapid Urease Test (RUT)

RUT requires MIU (Motility Indole Urea) medium. Test simultaneously checks for the production of indole, Urea hydrolysis and bacterial motility.

Composition of MIU base:

Ingredients	**Amount**
Proteose Peptone Difco	10.0g
Beef extract	5.0g
NaCl	5.0g
KH_2PO_4	2.0g
Phenol red solution (1/500)	6 ml

Agar	3.0g
Distilled Water	900 ml

PH= 6.8-6.9

Preparation of Urea solution:

Eight gram (8g) Urea powder was dissolved in 10 ml DW to make 80% urea solution. Then urea solution was sterilized using filtered using milipore filter.

MIU media preparation:

1.8g MIU base powder was dissolved in 95 ml distilled water in a conical flask

|

Then heated to melt all the ingredients

|

MIU base medium is autoclaved at 121^{o}C under 15 lb/in^{2} for 15 minutes and then cooled to about 50^{0}C

|

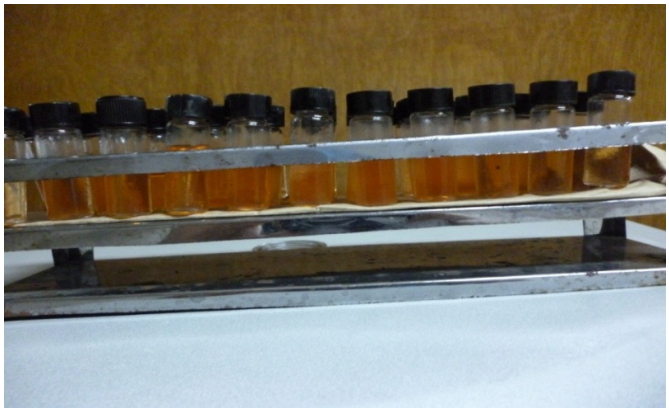
5 ml urea solution was added to 95 ml MIU base medium

|

Then sorted into small bottle before hardening

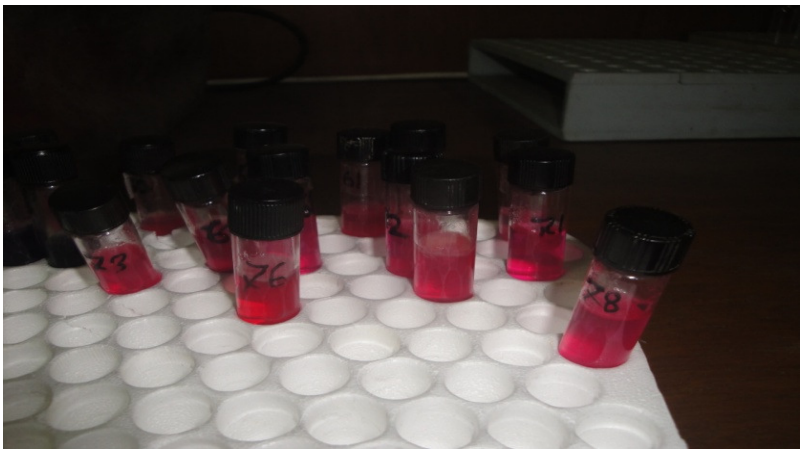
|

Final medium was semi-solid and kept in refrigerator



MIU Medium sorted in small bottle

RUT procedure: One piece of antral biopsy is placed into semi solid MIU medium and then incubated overnight at 37^0C. Positive result changed the color of medium from yellow to pink.



Appendix 4

ELISA Procedures:

1. Assay Procedure of IgG ELISA

Reagent Preparation

Wash Buffer dilution----1 vol. Wash Buffer: 19 vol. DW

Sample dilution

Prepare 1:40 dilution for test samples, all six *H. pylori* standards, low control, and high control by adding 5 µl of the sample to 200 µl of sample diluent.

Assay Procedure of IgG ELISA

Secure the desired number of coated wells in the holder

↓

For the reagent blank, dispense 100µl sample diluent in A1well position

↓

Dispense 100 µl of diluted sera, 1 standard, and controls into the appropriate wells

↓

Tap the holder to remove air bubbles from the liquid and mix well for 10 seconds.

↓

Incubate at room temperature for 30 minutes

↓

Remove liquid from all wells

↓

Rinse and flick the microtiter wells 4 times with diluted wash buffer and then one time with distilled water

↓

Dispense 100 µl of enzyme conjugate to each well

↓

Mix gently for 10 secoonds.

↓

Incubate at room temperature for 30 minutes

↓

Remove enzyme conjugate from all wells

↓

Rinse and flick the microtiter wells 4 times with diluted wash buffer and then one time with distilled water

↓

Add 100 µl of TMB Reagent to each well

↓

Mix gently for 10 seconds

↓

Incubate at room temperature for 20 minutes

↓

Add 100µl of Stop Solution to each well including the 2 blanks

↓

Mix gently for 30 seconds

↓

Read the optical density at 450 nm within 15 minutes

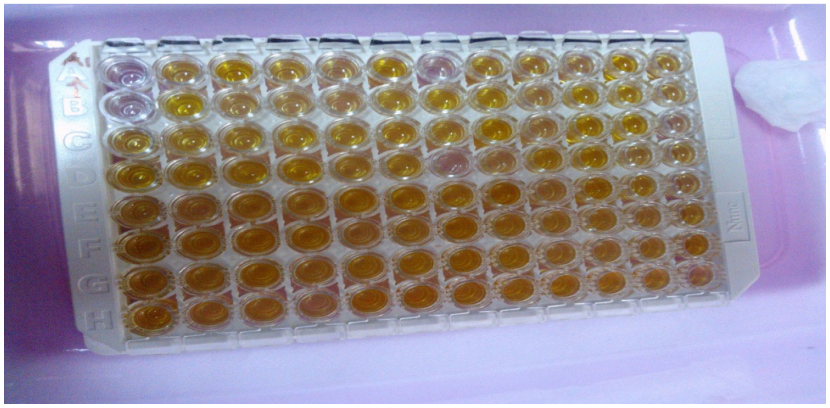


Figure: Anti-*H. pylori* IgG ELISA

II. Assay Procedure (IgA ELISA)

Select the required number of microtiter strips or wells and insert them into the holder

↓

Leave well A1 for substrate blank

↓

Dispense

100 µL of *Neg. Control* into well B1

100 µL of *Standard 1* into well C1

100 µL of *Pos. Control* into well D1

↓

100 µL of each diluted sample into appropriate wells.

↓

Incubate for 60 minutes at 37 °C covering wells with foil

↓

Briskly shake out the contents of the wells.

↓

Rinse the wells 5 times with diluted *Wash Solution*

↓

Strike the wells sharply on absorbent paper.

↓

Dispense 100 µL Enzyme Conjugate into each well, except A1.

↓

Incubate for 30 minutes at RT covering the wells

↓

Briskly shake out

↓

Rinse the wells 5 times with diluted *Wash Solution*

↓

Strike the wells sharply on absorbent paper

↓

Add 100 µL of Substrate Solution into all wells

↓

Incubate for exactly 15 minutes at RT in the dark

↓

Add 100 µL of *Stop Solution* to each well

↓

Blue color turns into yellow

↓

Read the OD at 450/620 nm within 30 minutes

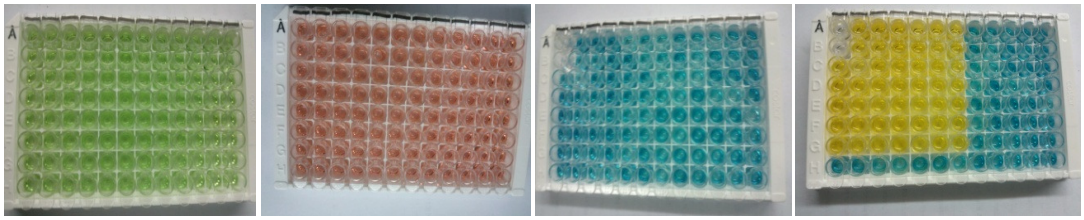


Figure:Anti- ***H. pylori*** **IgA ELISA**