# IDENTIFICATION OF *Helicobacter pylori* ANTIGENIC PROTEINS FROM ISOLATES OF PATIENTS WITH GASTRIC PATHOLOGIES

**By**

**LEE CHUN WEI**

**Thesis submitted in fulfillment of the requirement for**

**Masters of Science**

**JULY 2011**

## ACKNOWLEDGEMENT

I would never have been able to finish my dissertation without the guidance of my supervisors, help from staff, friends, and support from my family.

I would like to express my deepest gratitude to my supervisor, Dr. Amutha Santhanam, for her excellent guidance, caring, patience, and providing me with an excellent atmosphere for doing research. I would like to thank my co supervisor Prof Rahmah Noordin, who is generous in sharing her experiences and guidance, and patiently corrected my writing and financially supported my research.

I would also like to thank Dr. Nagarajan and all the clinic staff from Seberang Jaya Hospital endoscopy unit, for helping me in sample collection for the past several years for and teaching me techniques and knowledge related to this research. And most of all, thanks for being my friend and keep me cheerful during clinic sessions.

I would also like to thank the research staff of INFORMM and my colleagues. Especially to Puan Sabariah Osman, Puan Norhalida Hashim and Puan Maimunah Ahmad, for being so supportive and helpful during my course of research and not forgetting, to my dear colleagues, Akbar Khalipour, Ahmad Munir Mohamad Tan Sin Yee, Khoo Teng Kew, Tan Aik Aun, Izzati Zahidah and those whose name I didn't manage to mention here, thank you for making research a enjoyable time for me.

I would also like to thank the administration staff of INFORMM Penang, especially to Mr Irwan Effendy, Mr Mohd Azizi Ahmad, Mr Adli Akbar, Mr Mohd Nor Azam Zainol, and Miss Kamini for helping me directly and indirectly in my research.

I would also like to thank my parents, my grandparents, and my brother. They were always my greatest source of encouragement and inspiration.

This study was funded by Research University Grant No: 1001/PPSP/812016-2, without this funding, it is impossible to carry out this study. I would also like to thank USM Fellowship for supporting me for my postgraduate studies.

Finally, I would like to thank God. He has always been there with me through good and bad times. Thank you God you giving me the wisdom and strength for completing this dissertation.

## TABLE OF CONTENTS

**CHAPTER 2 MATERIALS AND METHODS**

**LIST OF TABLES**

**CONTENTS**

**PAGE**

## LIST OF FIGURES

**CONTENTS** **PAGE**

## LIST OF ABBREVIATIONS

| | |
|---|---|
| *cag* pathogenicity island | *cag* PAI |
| Cytotoxin-associated gene products A | CagA |
| Deoxyribonucleic acid | DNA |
| Polymerase Chain Reaction | PCR |
| Sodium-Dodecyl-Sulfate Polyacrylamide gel electrophoresis | SDS-PAGE |
| Vacuolating cytotoxin A | VacA |
| Heat shock protein B | HspB |
| Urease subunit C | UreC |
| Flagellin subunit A | FlaA |
| Flagellin subunit B | FlaB |
| Ornithine decarboxylase enzyme | ODC |
| Interleukin | IL |
| Kilo-Dalton | kDa |
| Degree Celsius | °C |
| Positive | † |
| Pound per square inch | psi |
| Negative | - |
| Enzyme-linked immunesorbent assay | ELISA |
| Immunoglobulin A | IgA |
| Immunoglobulin G | IgG |
| Immunoglobulin M | IgM |

| | |
|---|---|
| Excretory secretory antigen | ESA |
| Surface associated antigen | SAA |
| Toll-like receptors | TLR |
| Oesophageal Gastro-Duodenal Scope | OGDS |
| Voltage | V |
| Millimeter | mm |
| Micrometer | µm |
| Microgram | µg |
| Milligram | mg |
| Gram | g |
| Kilogram | Kg |
| Microliters | µl |
| Milliliter | ml |
| Liter | L |
| Morality | M |
| Millimolar | mM |
| Nanometers (wave length) | nm |
| Optical density | OD |
| Colony forming unit | CFU |
| Normality | N |
| Gravitation constant | *x g* |
| Volume | v |
| Weight | w |

| | |
|---|---|
| Times | X |
| American Type Culture Collection | ATCC |
| Tryptic Soy Agar with 5% sheep blood | TSA |
| Roswell Park Memorial Institute medium | RPMI |
| Dulbecco's Modified Eagle medium | DMEM |
| Bovine serum albumin | BSA |
| Fetal bovine serum | FBS |
| Hydrochloric acid | HCl |
| Sodium Hydroxide | NaOH |
| Hydrogen peroxide | $H_2O_2$ |
| Phosphate buffered saline | PBS |
| Sodium Chloride | NaCl |
| Potassium chloride | KCl |
| Disodium hydrogen phosphate | $Na_2HPO_4$ |
| Potassium dihydrogen phosphate | $KH_2PO_4$ |
| Tris buffered saline | TBS |
| Ethylenediaminetetraacetic acid | EDTA |
| (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid ) | HEPES |
| Phenylmethanesulfonylfluoride | PMSF |
| Ethidium bromide | EtBr |
| Sodium bicarbonate | $NaHCO_3$ |
| Sodium carbonate | $Na_2CO_3$ |
| 2,2'-Azino-d-[3-ethylbenthiazoline sulfonate] | ABTS |

| | |
|---|---|
| Tris-Borate-EDTA buffer | EDTA |
| Sodium-dodecyl-sulfate | SDS |
| Ammonium persulfate | AP |
| Carbon dioxide | $CO_2$ |
| Oxygen | $O_2$ |
| Nitrogen | $N_2$ |
| Magnesium Chloride | $MgCl_2$ |
| Deoxyribonucleotide mix | dNTP |
| *Thermus aquaticus* | Taq |
| Base pairs | bp |
| Kilo base pairs | Kb |
| Ultra violet radiation | UV |
| Horseradish-peroxidase | HRP |
| Cut-off optical density value | COV |
| Tetramethylethylenediamine | TEMED |

**PENGENALPASTIAN PROTEIN ANTIGENIK *Helicobacter pylori* DARIPADA ISOLAT PESAKIT YANG ADA PATOLOGI GASTRIK**

## ABSTRAK

*Helicobacter pylori* ialah bakteria gram negatif, berbentuk spiral, mikroaerofil, yang berkolonisasi pada bahagian atas apikal sel epitel perut dan lapisan mukosa manusia. Jangkitan *H. pylori* telah dikaitkan dengan pelbagai penyakit gastrik dengan spektrum klinikal yang luas, daripada kanser perut hingga ulser pada saluran pencernaan. Patogenisiti jangkitan bergantung pada virulens strain, kerentanan hos dan faktor persekitaran. Dengan itu, adalah sangat penting untuk memahami patogenesis penyakit ini. Kajian untuk mengenal pasti protein antigenik *H. pylori* memungkinkan pembangunan ujian diagnostik bagi patogen ini.

Beberapa media telah dinilai untuk tujuan mengisolasi dan mengkultur *H. pylori.* Tiga isolat klinikal digunakan, dan *H. pylori* ATCC 700824 merupakan strain rujukan. Untuk medium cecair, medium Infusi Otak Jantung ('Brain Heart Infuison') dan medium Tryptic Soya dengan 10% serum janin lembu diuji untuk mengenal pasti medium yang memberikan pertumbuhan mikrob yang lebih tinggi (cfu). Sementara itu, perbandingan antara medium pepejal yang dilengkapi dengan serum atau darah iaitu Agar Tryptic Soya (TSA), Agar Columbia, Agar Eugon dan Agar Infusi Otak dan Jantung (BHIA), menunjukkan bahawa medium dengan suplemen darah memberikan cfu lebih tinggi. Medium komersial, TSA dengan 5% darah biri-biri, telah menghasilkan pertumbuhan terbaik antara medium pepejal yang diuji.

Dalam kajian ini, biopsi sampel perut adalah daripada pesakit yang telah menjalani skop Kerongkong-Gastro-Duodenal (*Esophageal-Gastro-Duodenal*). Daripada 564 sampel biopsi yang diperoleh; 50 isolat *H. pylori* telah berjaya diisolasi daripada pesakit dengan pelbagai patologi gastrik. Keputusan penyaringan PCR menunjukkan bahawa semua isolat adalah positif bagi gen Ure C, sedangkan hanya 42 isolat yang positif untuk gen Cag A.

Satu 'in-house' anti-*H. pylori* ESA-IgG ELISA telah dibangunkan dan dibandingkan dengan kit komersil, iaitu EIAgen *H. pylori* IgG (Adaltis, Itali) dan keputusannya menunjukkan bahawa sensitiviti dan spesifisiti kedua ujian adalah sama. Empat kumpulan sampel serum digunakan, ia berdasarkan kepada keputusan kultur biopsi dan 'in-house' *H. pylori* IgG-ELISA: Kumpulan I: kultur positif dan seropositif; Kumpulan 2: kultur negatif dan seronegatif; Kumpulan 3: sihat dan seronegatif, dan Kumpulan 4: penyakit lain dan seronegatif. Tiga isolat yang berbeza yang berasal daripada patologi gastrik yang berbeza telah digunakan untuk ekstrasi protein, iaitu SJI002 dari gastroduodenitis, SJI016 dari ulser perut, dan SJI017 dari ulser duodenum. Dua penyediaan protein digunakan iaitu Antigen Ekskretori Sekretori (ESA) dan Antigen Perkaitan Permukaan (SAA). SDS-PAGE dan blot Western telah dijalankan ke atas ESA dan SSA, dengan menggunakan sampel serum yang dinyatakan di atas.

Satu siri jalur antigenik telah dikenal pasti sebagai protein yang mempunyai potensi diagnostik. Dengan menggunakan sediaan ESA, tiga jalur dikenal pasti daripada isolat SJI002 iaitu 17 kDa, 29 kDa dan 57 kDa. Daripada isolat SJI016, empat jalur dikenal pasti iaitu 13 kDa, 17 kDa, 29 kDa dan 41 kDa, dan daripada isolat SIJ017 tiga jalur telah dikenal pasti iaitu 13 kDa, 17 kDa dan 50 kDa. Dengan

menggunakan sediaan SAA, tiga jalur antigenik dikenal pasti daripada isolat SJI002 dan SJ1016 iaitu 13 kDa, 29 kDa dan 57 kDa. Manakala daripada isolat SIJ017, empat jalur dikenal pasti iaitu 13 kDa, 17 kDa, 29 kDa dan 57 kDa. Daripada keputusan yang diperolehi, dapat dilihat terdapat jalur antigenik yang dikongsi antara sediaan antigen dan antara isolat. Kajian ini telah berjaya mengenal pasti panel jalur antigenik yang berpotensi untuk digunakan bagi pembangunan ujian diagnostik bagi jangkitan *H. pylori* pada masa hadapan. Oleh sebab isolat yang digunakan adalah daripada pesakit tempatan, antigen yang dihasilkan dijangka sesuai untuk digunakan bagi mendiagnos pesakit di Malaysia.

**IDENTIFICATION OF *Helicobacter pylori* ANTIGENIC PROTEINS FROM ISOLATES OF PATIENTS WITH GASTRIC PATHOLOGIES**

## ABSTRACT

*Helicobacter pylori* is a gram negative, spiral-shaped, microaerophilic bacterium, which colonizes the apical side of human gastric epithelial cells and mucous layer. *H. pylori* infections are associated with various gastric diseases with wide spectrum of clinical outcomes, ranging from gastric cancer to ulceration of the gastrointestinal tract. The pathogenicity of the infection depends on the strain virulence, host susceptibility and environmental co-factors. Thus it is very important to understand the pathogenesis of the disease. Identification of the antigenic proteins of *H. pylori*, may eventually lead to the development of diagnostic test for this pathogen.

Several media were evaluated for isolation and propagation of *H. pylori.* Three clinical isolates were used and ATCC 700824 strain were employed as reference. For liquid medium, Brain Heart Infusion broth and Tryptic Soy broth with 10% fetal bovine serum were tested and the former was found to give higher microbial growth (cfu). Meanwhile comparisons between serum or blood supplemented solid media i.e. Tryptic Soy Agar (TSA), Columbia Agar, Eugon Agar and Brain Heart Infusion Agar (BHIA), showed that the media with blood supplement gave higher cfu. The conventional agar medium, TSA with 5% defibrinated sheep blood, produced the best growth among the solid media.

In this study, stomach biopsy samples were from the patients who underwent Esophageal-Gastro-Duodenal scope. Out of 564 biopsy samples, 50 isolates of *H. pylori*

were successfully isolated from patients with various gastric pathologies. PCR screening showed that all isolates were positive for *Ure* C gene, whereas only 42 isolates were positive for *Cag* A gene.

An in-house anti-*H. pylori* ESA-IgG ELISA was developed and compared with a commercially available EIAgen *H. pylori* IgG kit (Adaltis, Italy) and the results showed similar sensitivities and specificities. Four groups of serum samples were used, they were categorised according to the results of biopsy culture and the in-house IgG-ELISA: Group I: culture positive and seropositive; Group 2: culture negative and seronegative; Group 3: healthy and seronegative, and Group 4: patients with other diseases and seronegative. Three different isolates which originated from different gastric pathologies were used for protein preparation, namely SJI002 from gastroduodenitis, SJI016 from gastric ulcer, and SJI017 from duodenal ulcer. Two different protein preparations were used i.e. Excretory Secretory Antigen (ESA) and Surface Associated Antigen (SAA). ESA and SAA were then subjected to SDS-PAGE and Western blot analysis using the above serum samples.

A series of antigenic bands were identified as proteins with potential diagnostic value. For ESA, three bands were identified from isolate SJI002 namely 17 kDa, 29 kDa and 57 kDa. From isolate SJI016, four bands were identified namely 13 kDa, 17 kDa, 29 kDa and 41 kDa; and from isolate SIJ017 three bands were identified namely 13 kDa, 17 kDa and 50 kDa. Using SAA three antigenic bands were identified from isolates SJI002 & SJI016 i.e 13 kDa, 29 kDa and 57 kDa. From isolate SIJ017, four bands were identified i.e 13 kDa, 17 kDa, 29 kDa and 57 kDa. As can be observed there were some common bands between the antigen preparations and among the isolates.

Thus this study has successfully identified a panel of antigenic bands that are potentially very useful for future development of diagnostic test for *H. pylori* infection. Since these isolates were from local patients, the antigens derived from them would be very suitable to be used for diagnosis and screening of Malaysian Patients.

# CHAPTER 1
# INTRODUCTION

## 1.1 General introduction

*Helicobacter pylori* is a gram negative, spiral-shaped, micro-aerophilic, flagellated slow growing bacteria. It colonizes the apical side of human gastric epithelial cells and mucous layer (Benoit *et al*., 2004). It was first isolated in 1982 by Marshall and Warren, and found to be probably the most chronic bacterial infection of mankind (Rieder *et al*., 2005), infecting almost half of the human population especially in developing countries (Malaty, 2007). *H. pylori* infections are associated with various gastric diseases; among them the most common is gastritis, and is also implicated in more severe gastric diseases including chronic atrophic gastritis (a precursor of gastric carcinomas), peptic ulceration, duodenal ulcer and mucosa-associated lymphoid tissue lymphomas (Jungblut *et al*., 2000). The bacterium was also classified as class I carcinogen by the World Health Organization (Frenck and Clemens, 2003) after a few studies proved that it has the capability to cause and induce gastric carcinoma in animal models (Giannakis *et al*., 2008). *H. pylori* is also associated with some other associated diseases apart from gastric pathologies such as asthma (Franceschi and Gasbarrini, 2007), atherosclerotic heart disease (Franceschi and Gasbarrini, 2007). Consequently the early identification/detection and treatment of *H. pylori* infection can prevent or reduce morbidity and mortality rates of patients (Chisholm and Owen, 2008).

There is a wide spectrum of clinical outcomes induced by chronic gastritis caused by *H. pylori*, ranging from ulceration to gastric cancer of the gastrointestinal

tract. The severity of the infection depends on the strain virulence, host susceptibility and environmental co-factors. Some of the infected patients are symptomatic, whereas, some will remain asymptomatic for life (Malaty, 2007). However, 6-20% of the infected individuals tend to develop duodenal ulceration and a small proportion of them will eventually advance to gastric cancer. Nevertheless, duodenal ulcer and gastric cancer vary greatly in their symptoms and pathology, hence, the differences in pathogenesis is a major factor that greatly governs the different patterns of gastritis (Lochhead and El-Omar, 2007). In view of the fact of *H. pylori* infection can cause such serious diseases, it is very important to understand the pathogenesis of the disease with the intention for identifying those patients which are at high risk of developing cancer (Brooks *et al*., 2004). During the process of *H. pylori* infection, the antigens secreted from the bacterium elicit strong humoral immune responses which are characterized by the infiltration of the mucosa layer by neutrophiles, lymphocytes and macrophages. These antigens can thus be regarded as prospective candidates that may serve as infection biomarkers (Robinson *et al*., 2007).

Various studies on *H. pylori* genome had suggested that the DNA of the species is extremely diverse, leading to differences in genotypes and phenotypes. This, in addition to genetic physiology and immunological factors in various combinations in human host, will thus likely cause varying infection outcomes (Proenca Modena *et al*., 2007). So, it is very important to understand how these various factors play a role in eliciting and causing disease in human host and its impact on the outcome of infection. Since proteins and antigens are the main cause of the disease and infection, it is vital to understand the interaction of these proteins with the human host and further characterize them.

## 1.2 The taxonomy *Helicobacter* and morphology

The genus *Helicobacter* belongs to the phylum Proteobacteria, class Epsilonproteobacteria, family Helicobacteraceae and order Campylobacterales (On, 2001; Windsor & O'Rourke, 2000). *Helicobacter* infection is not only limited exclusively to humans, however it was found in various mammalian animals including domestic animals such as swine, canine, sheep and etc. There are few reported studies on species other than *H. pylori* such as *H. acinonychis* (cheetah), *H. bizzozeronii* and *H. canis* (canine), *H. felis* (felines), *H. trogantum* (swine and sheep), *H. bilis* (mouse) and many other animals. In human, there are also different strains of *Helicobacter* bacterium isolated and reported through various studies, for instants *H. pylori* (gastric mucosa), *H. cinaedi* (feaces), *H. pullorum* (feaces), and a few unclassified strains such as *R53* (gastric mucosa), and *Helicobacter. sp. taxon 8* (feaces). However to what extent these strains of *Helicobacter* implicated in human disease is still unknown (Mikkonen *et al*., 2004).

*H. pylori* is a spiral shape, gram negative bacterium, about 3 μm long with a diameter of about 0.5 μm and is microaerophilic (Olczak *et al*., 2002). Under the microscope, the bacterium appears to be a corkscrew shape bacterium with a spiral shape flagella attached at its rear end. This is partly why it is called "helicobacter" and the term pylori refers to their favorite fortress. It has the ability to colonize and form a biofilm on the stomach epithelium tissues and cause an invasive infection. The bacterium is also capable of converting from a spiral, viable form to a possibly viable, but nonculturable coccoid form to favor its survival. The coccoid form is capable of adhering to gastric epithelia cells *in vitro*.

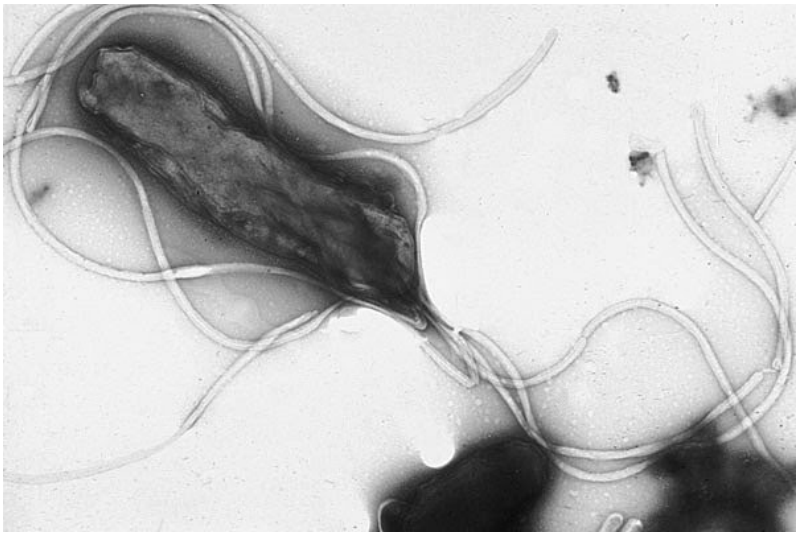

**Figure 1:** Microscopic appearance of *Helicobacter pylori*

Source :http://sites.google.com/site/scienceprofonline/infectiousdiseasescausedbybacteria

## 1.3 Pathogenesis of infection

A pathogen is distinguished from a non-harmful pathogen by focusing on its virulence factors. *H. pylori*'s ability to reside and persist for many years within a host is even more important than its ability to damage the host tissues. Most of the pathogenesis seen in *H. pylori* are due to strain virulence, the host mechanism to the bacterium and not because of the toxicity mediated by the bacterium itself (Andersen *et al*., 1997).

### 1.3.1 Virulence factors

Virulence factors had been extensively studied in an effort to associate bacterial phenotype with specific manifestations of disease, and to explicate the mechanisms of pathogenesis. To date, there are vast arrays of virulence factors identified and reported through various studies and some are frequently associated with the most serious clinical outcomes. For instants, *cag* pathogenicity island (cag PAI) encodes a type IV secretion apparatus (Censini *et al*., 1996; Argent *et al*., 2008). The *cagA* gene encodes a protein (*CagA)* that is injected into the cytoplasm of the host cell and induces cellular morphological alterations, proliferation, adhesion and apoptosis. The *cag*PAI also contains a gene known as *cagE* or *picB* that encodes a secretory protein that is required for the induction of interleukin (IL)-8 production by epithelia cells (Proenca Modena *et al*., 2007). The polymorphism of *Cag*A is also strongly associated with *H. pylori* pathogenicity. In recent study, it is found that the increase in number of EPIYA-C fragments in the *Cag*A gene will increase the risk of developing gastric cancer (Basso *et al*., 2008). Patients with *Cag*A positive strains are generally found to have

more severe symptoms than patients infected with *Cag*A negative strains (Umit *et al*., 2009).

Another important virulence factor of *H. pylori* is a 95 kDa secreted protein denominated VacA that induces apoptosis partly by forming pores in the mitochondria membrane, thereby allowing the efflux of cytochrome C. This protein also forms pores in the cytoplasmic and vacuole membranes, increases the paracellular permeability and causes the vacuolization of host epithelia cells and produces immonosuppression by blocking antigen presentation to T cells and the maturation of macrophage phagosomes (Terebiznik *et al*., 2006). The signal sequence (*s1a, s1b, s1c, s2*) and/or the middle region (*m1, m2*) of *VacA* varies considerably among *H. pylori* strains and strains with the *s1m1* genotype are more pathogenic (Blaser and Atherton, 2004). The presence of i1 or i2 "intermediate region" also greatly increases the risk of developing peptic ulcer in patients (Basso *et al*., 2008). In a recent study, it is also found that both *Vac*A and *Cag*A down regulates each other's activity and allow interaction of the bacterium with the gastric epithelia cells by preventing excessive damage *(*Argent *et a*l., 2008).

Despite all the above significant discoveries, there are still a vast amount of true and hypothetical virulence factors that are widely published that plays either major or minor role in manifestation of disease which had not yet been extensively studied, such as neutrophil-activating protein (NapA), and cochaperone HspA (GroES) (Kimmel *et al*., 2000) and etc. These virulence factors might have potential in predicting the manifestation of specific pathologies (Andersen and

Espersen, 1992), which could have significant diagnostic values, awaiting to be explore.

### 1.3.2 *H. pylori* binding factors

When *H. pylori* infects a host, it must have an ability to bind to the mucosa layer and gastric epithelium in order to establish an infection. But to date, the binding mechanism of the bacterium is still not fully understood. A variety of different binding sites are present on *H. pylori*, which enables the bacterium to bind to the receptors on the surface of the epithelium cells and hence, enabling *H. pylori* to bind to the surface of the stomach and start its colonization (Wadstrom *et al*., 1994).

Some binding factors that have been reported are HSP-60 which has the ability to bind to human gastric carcinoma cells (MKN45) *in vitro* (Yamaguchi *et al*., 1997b); N-acetylneuraminyllactose- binding fibrillar haemagglutinin which specifically binds to GM3 gangliosides and sulphatides on the surface of the host cells (Saitoh *et al*., 1991) and a laminin-binding factor (Valkonen *et al*., 1994). These indicate that the adhesion of *H. pylori* to human gastric epithelial cells might be multifunctional in disease development. Apart from that, the binding also ensure that *H. pylori* are not washed away with the peristaltic movement inside the stomach during digestion.

### 1.3.3 Motility of *H. pylori*

*H. pylori* is a flagellated bacterium which have the ability of being motile. They can roam freely in the lumen of the stomach before binding to the epithelial cells and causing an infection (Eaton *et al*., 1991). Motility of the *H. pylori* bacterium is governed by two major flagella forming genes, *flaA* and *flaB*. These genes encodes for flagellin subunits namely FlaA and FlaB, which are coexpressed in different amounts which makes up the functioning flagella of the bacterium (Josenhans *et al*., 1995). *H. pylori* demonstrate high level of motility in animal models, even in highly viscous environment than other enteric bacteria, such as salmonellae or *Escherichia coli* (Hazell *et al*., 1986).

Ability of *H. pylori* to colonize and establish an infection greatly depends on its ability to be motile. Few animal models studies in relation to human had concluded that motility plays a major role in colonizing and persistence of the bacterium on the stomach epithelium, and hence, motility is considered as a virulence factor (Josenhans *et al*., 1995).

### 1.3.4 Urease activity of *H. pylori*

Urease is one of the most important virulence factors of *H. pylori*. The marked urease activity of *H. pylori* gives the organism the ability to colonize the acidic environment of the stomach by providing an alkaline microenvironment. Urease was the first virulence factor of *H. pylori* used for diagnostic purposes in gastric pathology (Eaton *et al*., 1991). The urease catalyzes the degradation of urea to ammonia and bicarbonate. Ammonia alkalizes the environment, leading to neutralization of the acid fluid in the stomach, which enable the survival of the bacterium in the stomach (Hazell and Lee, 1986). When the hydrochloric acid concentration in the stomach reduces due to urease activity, it will stimulate the bacterium to colonize, and hence enabling the *H. pylori* to survive in the gastric mucosa permanently and causes disease (Stingl and De Reuse, 2005). Thus, urease is considered as an important virulence factor.

*H. pylori* also has the ability to produce permanent factors such as IL-1which can prevent the secretion of hydrochloric acid from the stomach parietal cells *(Saperas et al*., 1990); thus lowers the acid concentration in the stomach that enables the survival of *H. pylori* cells during the early phase of colonization. This enables *H. pylori* to colonize easily at the first stage of the infection.

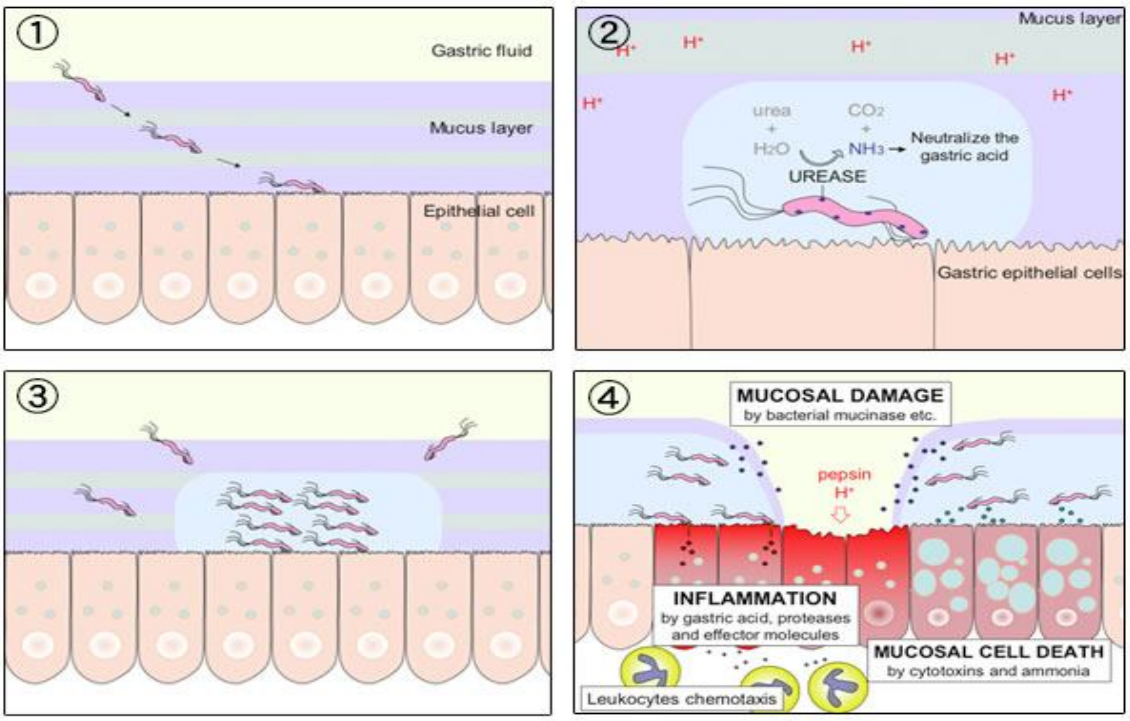


**Figure 2:** *H. **pylori*** invades epithelial cells of human host

Source:http://upload.wikimedia.org/wikipedia/commons/thumb/d/db/H_pylori_ulcer_diagram.png/800px-H_pylori_ulcer_diagram.png

*H. pylori* are able to attach to the epithelial cells of the stomach and duodenum which stops them from being washed out of the stomach. Once attached, the bacteria start to cause damage to the cells by secreting degradative enzymes, toxins and initiating a self-destructive immune response.

## 1.4 Mode of transmission of *H. pylori*

There are several hypotheses on the mechanisms involved in the mode of transmission of *H. pylori*; along with the postulation that human being is one of the sources of *H. pylori*. One hypothesis is that pathogen should be produced from the human stomach and appeared in gastric juice, saliva or dental plaque so that it can be easily transmitted to others through various routes. There are many different modes of transmission of *H. pylori* which is not fully understand, and hence, further research and investigation is needed to verify the actual path of dissemination of *H. pylori* (Malaty, 2007).

### 1.4.1 Transmission through direct contact

Human seems to be the only reservoir of *H. pylori* which spread from person to person via oral-oral, fecal-oral or gastro-oral routes (Kim *et al*., 2000). Various studies had demonstrated that *H. pylori* is present on human dental plaque (Li *et al*., 1996) which suggest that the prevalence of this bacterium is high in human's saliva and it is spread via oral to oral route through various activities such as kissing, during meals and so on. But various investigators have found the organism with greater or lesser frequency in oral samples in different studies (Leimola-Virtanen *et al*., 1995). There is also a report stating that the oral cavity acts as a permanent reservoir for *H. pylori* (Leung et al., 1999b). As for fecal-oral route, both *H. pylori* bacterium and DNA are detected in stool culture and PCR respectively. However, the sensitivity for detection of *H. pylori* in stool is low in comparison with direct histological staining and culture of biopsy

samples (Kolho *et al*., 2006), yet there is still no solid scientific explanation why in certain cases, *H. pylori* DNA is detected in fecal material while the gastric biopsy sample was negative for culture, histological stain and PCR (Li *et al*., 1996).

### 1.4.2 Waterborne transmission

*H. pylori* is also known to be transmitted through water, due to fecal contamination, and this might be an important source of infection, especially in parts of the world in which untreated water is frequently consumed (West *et al*., 1990). And some research findings concluded that children who use external water sources have 3 times higher risk than children who consume treated water (Klein *et al.*, 1991). However, *H. pylori* was also reported to be found in treated tap water (Fan *et al*., 1998). The use of municipal water has also correlated with *H. pylori* infection in populations from both low and high socioeconomic status. In addition, swimming in stream water in the rural area of most of the developing country also increased the rate of infection of *H. pylori* (Lu *et al*., 2002). More studies should be carried out to further shed light on this mode of transmission.

### 1.4.3 Transmission of *H. pylori* through animals

Besides human, *H. pylori* also have been isolated from other animals. *H. pylori* isolated from domestic animals such as cats, dogs and sheep have been widely reported (Benoit *et al*., 2004; Eaton *et al*., 1991; Hanninen *et al*., 1995; Harbour and

Sutton, 2008; Quaglia *et al*., 2007). However there is no solid evidence on the transmission of *H. pylori* from animals to humans are imminent. The most recent reservoir suggested for *H. pylori* is housefly (Brown *et al*., 2001). However, evidence is lacking that *H. pylori* can be transmitted to human from flies that have been on contact with *H. pylori*-infected feces. Nevertheless, the hypothesis is appealing since flies are known to carry many other infectious diseases.

## 1.5 Gastric pathologies and associated diseases

### 1.5.1 Gastric pathologies

*H. pylori* is commonly associated with gastric pathologies such as peptic ulcer diseases (PUD) which includes gastric, gastric ulcer (GU), duodenal ulcer (DU), atrophic gastritis, gastric cancer (GC) and mucosa-associated lymphoid tissue lymphoma (Blaser, 1998). The strong relationship between *H. pylori* infection and peptic ulcer diseases has been studied and proven extensively, and is accepted that the organism is the major causative agent but not the only cause behind these diseases globally (Blaser and Atherton, 2004). By eradicating the bacterium in the stomach, it can alter the natural course of peptic ulcer disease by drastically reducing its recurrence rate in treated patients, as compared to untreated patients (Delaney *et al*., 2008).

The mechanism by which *H. pylori* induces peptic ulcer disease (PUD) is incompletely understood but various findings suggested that the development of the disease involves a combination of genetic predisposition of the host, virulence factors

of the organism such as *Cag*A and *Vac*A, mechanical damage to the mucosa which is cause by unhealthy habits such as drinking and smoking, and finally, the alterations of gastric duodenal secretions (Giannakis *et al*., 2008; Kandulski *et al*., 2008; Love, 2008; Proenca Modena *et al*., 2007). Usually, when *H. pylori* invades stomach, it will express an active form of vacuolating cytotoxin (VacA) and a protein secretory apparatus called Cag (cytotoxin-associated gene products) that stimulates the host inflammatory responses (Viala *et al*., 2004). Cag + strains interact more closely with epithelial cells and induce release of pro-inflammatory cytokines, and thereby increase inflammation (Kandulski *et al*., 2008).

*H. pylori* is a genetically diverse organism. The genetic variation of the organism differs from strain to strain and from region to region, and there are no absolute standards stating that certain strains are more 'ulcerogenic' than other strains, since other factors such as host and environment played important roles as well in the infection outcome (Majewski and Goodwin, 1988).

### 1.5.2 Non-ulcer dyspepsia

Non-ulcer dyspepsia comprises of a collection of assorted symptoms, including ulcer-like and reflux-like symptoms. There are numerous causes suggested for non-ulcer dyspepsia, including lifestyle factors, stress, altered visceral sensation, increase serotonin sensitivity, alterations in gastric acid secretion, gastric emptying, and *H. pylori* infection (Veldhuyzen van Zanten and Sherman, 1994). A recent review also highlighted the role played by psychosocial impairment in patients with non-ulcer

dyspepsia (Monteiro *et al*., 2001a). In a study linking *H. pylori* infection to non-ulcer dyspepsia, patients with the latter condition were twice as likely to be positive for the organism (Armstrong, 1996). Eradication of *H. pylori* cannot be considered as the standard of care in all patients with non-ulcer dyspepsia, because *H. pylori* infection is only one of the multifactorial etiologies of the disease manifestation.

### 1.6 Epidemiology

*H. pylori* is a common pathogenic bacterium infecting more than half of the world's population (Moayyedi, 2007). Human stomach, and oral cavity are said to be the biggest reservoir of the bacterium, and the transmission probably occurs via person-to-person route (Malaty, 2007). In developing countries, the prevalence rate of the bacterium is considerably much higher in comparison with developed first world countries. Within these regions, subgroups are found to have higher prevalence rate in comparison to the general population, and it is greatly affected by the variations in age, socioeconomic status, ethnic backgrounds and geographical location (Nowottny and Heilmann, 1990).

Recent findings show that there is a decreasing trend of prevalence in those countries with rapidly improving socioeconomic conditions and developed countries. As for developing Asian countries, the prevalence of *H. pylori* are declining as shown in both seroprevalence-based and endoscopy based studies (Goh, 1997). This condition is also observed in other regions globally as well, like North America, Western Europe and Korea (Torres, 2000). Despite the decrease in prevalence, there

has been an increase in gastric cancer in Asia (Leung *et al*., 2008) and this phenomenon had been carefully monitored to study the relationship between the decreasing prevalence and incidences of gastric cancer. A numbers of convincing findings carried out in that region confirmed that age is an important factor for susceptibility towards gastric cancer, and the rate of developing gastric cancer appears to be higher in developing countries than in developed countries (Giannakis *et al*., 2008; Kuipers *et al*., 1993a; Kuipers *et al*., 1993b)

The risk of an individual for developing chronic diseases associated with *H. pylori* varies; many case studies have discovered that, the risk increases significantly during childhood and the infection shows variatiosn among adults. Fundamentally, the infection of *H. pylori* occurs in early childhood (Das and Paul, 2007). Childhood risk factors that are important includes contaminated and uncooked vegetables, contaminated water delivery systems, mother breastfeeding with saliva-coated nipples, poor hygiene, sharing beds during childhood and pre-mastication of food by mother for their children (Frenck and Clemens, 2003; Ahmed *et al*., 2009). Hence, the related factors above explain why such increases in infection are inevitable due to certain lifestyle practices.

In northern peninsular Malaysia, there are relatively low prevalence rate of *H. pylori* among the population. It is postulated that the genetic inheritance where by the gene from infected host passed on to the next generation will prolong *H. pylori* associated diseases. The Chinese and Indian communities are originally immigrant races that may have brought the infection over from their home countries to Malaysia (Li *et al*., 1991). Besides that, it is also found that the prevalence of *H. pylori* infection

also increased with age across all ethnic groups and gender. Based on a previously published study, the Indian, Chinese and Bangladeshi community are found to have higher prevalence of *H. pylori* during their childhood as compared to the local Malay community (Uyub *et al*., 1994), and the main contributing factors reported were contaminated food via non hygienic means and different lifestyle practiced by the different ethnic groups. Besides that, the prevalence of *H. pylori* is also found to vary among different geographical regions in the same country. A study has documented different prevalence in *H. pylori* in northern peninsular Malaysia and one of the main contributing factors was the poor and unhygienic environment condition (Sasidharan *et al*., 2008; Uyub *et al*., 1994).

In addition, the infection of *H. pylori* acquired in childhood can lead to multifocal gastritis and the patients are thus predisposed to various gastric symptoms later in life (Rocha et al., 2003). The main causative factors for childhood exposure are in developing countries includes poor water supply system, poor sewage treatment and disposal and other unhygienic environmental conditions affecting those with low socio economic status (Ahmed *et al*., 2007). Senior citizen above the age of 50 are the most infected with *H. pylori* and such condition can be correlated with the lifestyle of Malaysians 50 years ago and they developed symptoms only later in life as the bacterium will need time to manifest the symptoms of the infection (Khan, 1998). However, the population of the new generation has benefited from the improvements in economics and hygienic levels of the country; this had led to the reduction in the prevalence rate of *H. pylori* among youngsters. It was reported that the seroprevalence rate among children had fallen drastically (10.3%) as compared to adults (50%) (Boey

*et al*., 1999) and it is believed that the improvement of life style and hygienic level is the main contributing factors behind this development. Similar studies performed in Malaysia (Raj *et al*., 2001) and Singapore (Kang *et al*., 1990; Vu and Ng, 2000) show that there are significant differences in ratio of prevalence of *H. pylori* with respective age group across all ethnics and gender. But in certain cases, age related physiological changes also may predispose the individual to the infection (Parsonnet *et al*., 1988). Essentially, in a multi-racial community, socioeconomic and social-cultural practices are being associated to the relative difference in prevalence of the bacterium (Ahmed *et al*., 2007).

In Europe, the estimated incidences of infection at childhood the age of 10 are considerably high, around 50% of the children were found to be infected with *H. pylori.* The risk factors are similar with those identified in Asian countries, ranging from contaminated food, water source and low socioeconomic status (Torres, 2000). Transmission from parent to child has also been implicated and in some cases (Rocha *et al*., 2003), child-to-child transmission was also observed (Goodman and Correa, 2000). Thus, it shows that transmission is common among people with close relationships.

## *1.7* Diagnosis of *H. pylori*

To date, many diagnostic methods have been developed for screening of *H. pylori* in patients. The samples are obtained from the patients via both invasive and non invasive methods; each has their advantages and disadvantage, taking into consideration factors such as ethics, feasibility, economy and accuracy of the diagnostic tests. Many diagnostic tests has been used for detecting *H. pylori* in patients, but over the years, more diagnostic methods have been developed which give better accuracy.

### 1.7.1 Invasive test

Conventionally, invasive test has been employed by physicians to diagnose *H. pylori* infection in patients through endoscopy. The biopsy taken from the stomach epithelia by means of endoscopy was used on various diagnosis procedures to detect the presence of the bacterium in patients (Rautelin *et al*., 2003). There are a few types of invasive methods that are widely employed in medical field they are namely, histopathology examination, culture and rapid urease test (Zuniga-Noriega *et al*., 2006). Histopathology staining remains one of the best methods for detection *H. pylori* infection, and together with the visual observation by endoscopy; it provide a consortium of information based on the pathological condition of the stomach (Makristathis *et al*., 2004). However endoscopy is considered as expensive, unpleasant for patients and need highly specialized and experienced endoscopists to perform, in some cases, endoscopy is too expensive to be performed as a routine diagnosis for

gastric patients. Sampling error may also occur and causes false-negative results (Mitchell and Megraud, 2002) that can lead to inaccurate diagnosis.

A recent study has found that rapid urease test is able to provide a fast and simple diagnosis, with high specificity, and relatively low cost (Zuniga-Noriega *et al*., 2006). The procedure is performed by placing a single biopsy specimen in a test vial containing rapid urease test solution, incubate at 37°C overnight and observe for conversion of colors, from yellow to pink, which is conclude as a positive test. The method is the most time efficient and cheap to used in routine diagnosis procedures (Yousfi *et al*., 1996).

Culture is one of the options for detecting the presence of *H. pylori*. This method is rather difficult as it requires tedious and demanding culture techniques, with many factors that must be controlled, as compared to other methods. Culture needs specialized conditions for specimen transportation and speed in processing of the samples to increase the probability of recovering the organism. The media used are rather expensive, coupled with special conditions for maintenance, and the length of time necessary to obtain a culture result is slow compared to other methods (Perez-Perez, 2000). However, one of the advantages of culture diagnosis is antibiotic susceptibility test; this test can be performed in cases of antibiotic resistance (Wadstrom *et al*., 1994).

PCR is also sometimes used in diagnosis of *H. pylori*. This method can either be invasive or non invasive, depends on the samples use. This method has been used successfully to detect the presence of *H. pylori* in biopsy specimens, water and feces

with sensitive, specific results with short period of time (Monteiro *et al*., 2001b; Lage *et al*., 1995; Brooks *et al*., 2004). But one of disadvantage is the need of specialized personal to perform the test.

### 1.7.2 Non-invasive test

As for non-invasive test, there are also a few well-recognized methods employed widely in diagnosis of *H. pylori* infection, which include serology and carbon-labeled urea breath test. The presence of antibodies specific towards *H .pylori* in serum, whole blood and saliva is tested using serological methods like ELISA and western blotting (Alkout *et al*., 1997; Faulde *et al*., 1991; Kimmel *et al*., 2000). Even though serology test gives high sensitivity and specificity towards *H. pylori,* but may not discriminate between asymptomatic, active or chronic infection and past infection due to persistence of circulating antibodies to *H. pylori* in the body (Blanco *et al*., 2008). These antibodies usually remain detectable in human body after successful eradication.

Urease breath test is a non-invasive test based on metabolism of ingested labeled urea to labeled carbon dioxide, and it is a highly predictive method for diagnosis of *H. pylori* infection. It is very useful for detection of active infection, however, false negative results are possible if patients have undergone recent treatment, but a valid urease breath test can be performed a week after discontinuing therapy (Monteiro *et al*., 2001a).

PCR is another alternative for non-invasive test which is used to detect the presence of *H. pylori* DNA in human samples like saliva, dental plaque and feces that

is capable of providing accurate results in a relatively short period of time (Monteiro *et al*., 2001b; Lage *et al*., 1995; Li *et al*., 1996).

## 1.8 **Diagnostic markers**

Currently, there are various diagnostic markers reported for *H. pylori* infection. These markers, some are commercialized, give great advantages in detecting and diagnosing *H. pylori* infection in patients.

The accuracy of these markers varies from test to test, depending greatly on type of sample and type of assay, thus some are no longer adequate to justify their use based on both clinical and economical aspects (Vaira and Vakil, 2001). However, there is a new range of markers that had been made into commercialized diagnostic kits and provide better efficiency in terms of both accuracy and economy. Diagnostic kits that utilize stool antigen and carbon labeled urease breath test (Manes *et al*., 2001a; Manes *et al*., 2001b) have opened new options in diagnosis of active *H. pylori* infection. It is suggested that these kits should replace serological test particularly in test and treatment strategies currently being recommended in clinical practices (Vaira and Vakil, 2001).

There are two types of markers used in diagnosing *H. pylori* infection. They comprise of DNA and protein markers. DNA markers are detected by the means of PCR and will give very sensitive results. PCR are done on samples such as tissue biopsy samples, stool and gastric juice, and PCR proves to have a good sensitivity and specificity towards all types of samples (Lage *et al*., 1995), but routine use of PCR for

diagnosis has also been proven to be problematic as inhibition is inevitable in certain samples such as stool (el-Zaatari *et al*., 1995; el-Zaatari *et al*., 1997), and if other bacterium contains homologous sequences, it will yield false-positive results, hence lowering its specificity (Sugimoto *et al*., 2009).. Despite all the disadvantages, PCR remains as a promising tool that enables the detection of antimicrobial resistance by detecting mutations correlated with various antibiotics (Sevin *et al*., 1998). Currently, PCR remains a research tool for diagnosing *H. pylori* infection.

Protein diagnostic markers are mainly detected by the means of serological testing. There are numerous immunoassays developed for detection of anti-*H. pylori* IgG, IgA and IgM antibodies produced during the course of infection. These antibodies can be detected using whole blood, serum, saliva, stool and urine (Manes *et al*., 2005; Sasidharan and Uyub, 2009a; Faulde *et al*., 1991; Miwa *et al*., 2001; Simor *et al*., 1996). The two most developed methods such as immunoblotting (Aucher et al., 1998; Haas et al., 2002; Keenan *et al*., 2000) and ELISA (Rautelin *et al*., 2003; Simor *et al*., 1996; Uyub *et al*., 1994) are widely used in clinical research. Detection of *H. pylori* infection markers via serology method is the easiest non-invasive approach to diagnose this infection, with some test requires only a few drops of blood from the finger and the results are available in less than 5 minutes. However serological diagnosis is still not well recognized because of the prevalence of *H. pylori* antibodies post-treatment (Rosenstock *et al*., 2000).

### 1.8.1 DNA markers for *H. pylori*

Various diagnostic markers have been discovered and used widely in clinical diagnosis and research of *H. pylori*. These markers focus mainly on the virulence factors of the pathogen. Diagnostic markers such as *vacA* encodes for a vacuolating cytotoxin that induces apoptosis in epithelia cells (de Bernard *et al*., 2004); while *cagA* codes for a cytotoxin A that promotes the alteration of cellular membrane and apoptosis of the epithelia cells (Shmuely *et al*., 2005; Censini *et al*., 1996). Other genes such as *iceA* and *ureC* (Chomvarin *et al*., 2008) are also routinely used to screen the presence of *H. pylori* infection in a patient. However, the most popular DNA diagnostic markers are *cagA, vacA and ureC*. These genes are routinely used both in clinical and lab for diagnostic purposes from clinical samples such as blood, biopsy tissues, feces, saliva, whole blood and cultures (Chisholm and Owen, 2008; Monteiro *et al*., 2001b; Brooks *et al*., 2004; Kim *et al*., 2000; Li *et al*., 1996). However, the specificity and sensitivity of the diagnosis varies, according to different sample preparations and various laboratory conditions (Sugimoto *et al*., 2009).

On the market, there are very few commercial DNA based diagnostic kits available for *H. pylori*, most of the tests are performed in specialized laboratories with trained personals, since the assay is prone to contamination without proper handling and facilities (el-Zaatari *et al*., 1997). Requirement for cold chain transport makes it not accessible to developing countries, hence, DNA based diagnostic of *H. pylori* is not as popular as protein based diagnostic kits.

### *1.8.2* **Protein markers for *H. pylori***

Protein based markers are very popular compared to DNA based markers, as it is manufactured into cheap user friendly forms, such as immobilized strips. Besides that, protein based diagnostic kits are also available in ELISA and latex agglutination test cards. Immunoblotting is also performed as a lab-based serological method. These assays detect anti-*H. pylori* antibodies in either fecal, whole blood, serum, urine, and stool (Sasidharan and Uyub, 2009a; Zuniga-Noriega *et al*., 2006; Simor *et al*., 1996; Glassman *et al*., 1990; Miwa *et al*., 2001).

In serology studies, various *H. pylori* proteins had been employed as infection markers for diagnosis. Markers such as CagA, VacA, HspB, FlaA, FlaB, UreC are most commonly used (Cremonini *et al*., 2004; Schumann *et al*., 2006). In addition, there are also several unidentified proteins such as 18, 39.5, 33 34 kDa which were reported to be of potential diagnostic values (Galmiche *et al*., 2000; Keenan *et al*., 2000; Andersen and Espersen, 1992; Haas *et al*., 2002).

Lately, there is also another array of markers that not only act as diagnosis markers, it can also be used to predict the outcome of the infection. For instance, heat shock protein 60 (HSP-60) is associated with adhesion of the *H. pylori* cells onto the epithelial cells, which can further accelerate the infection process of *H. pylori* (Yamaguchi *et al*., 1997a). CagA was also used to determine the relation between *H. pylori* infection and gastric cancer. If the gastric cancer patients was positive for IgG against CagA, *H. pylori* is probably the main cause of gastric cancer, hence, strains which are CagA+ are more pathogenic compared to CagA- strains. Therefore, more attention should be focused on eradication CagA+ strains in patients to prevent further