

**PRODUCTION OF RECOMBINANT UREASE
FOR SCREENING OF
Helicobacter pylori INFECTION**

by

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LIST OF ABBREVIATIONS

Amp	Ampicillin
AP	Alkaline Phosphates
bp	Base pair
BSA	Bovine Serum Albumin
dATP	Deoxyadenosine triphosphate
dH ₂ O	Distilled water
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleotide triphosphate
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra acetic acid
EIA	Enzyme Immunoassay
Ek	Enterokinase
ELISA	Enzyme Linked Immunosorbent Assay
IMAC	Immobilized metal affinity chromatography
IPTG	Isopropyl-beta-D-thiogalactopyranoside
kb	Kilobase
kDa	Kilodalton
LIC	Ligation Independent Cloning
MW	Molecular Weight
OD	Optical Density
PCR	Polymerase Chain Reaction
PVDF	Polyvinylidene fluoride
Rf	Relative mobility factor

SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium-Dodecyl-Sulphate Polyacrylamide Gel
TAE	Tris-Acetate EDTA
Taq DNA polymerase	<i>Thermus aquaticus</i> DNA polymerase
TEMED	N,N,N', N'-tetramethylethylenediamine
TSB	Tryptic Soy Broth
WHO	World Health Organization
MWCO	Molecular Weight Cut-Off
λ	Wavelength

**PENGHASILAN REKOMBINAN UREASE
UNTUK PENGESANAN JANGKITAN *Helicobacter pylori***

ABSTRAK

Helicobacter pylori mempunyai kemampuan untuk menyebabkan jangkitan pada gastrousus dan mengakibatkan penyakit pada pangkal usus seperti radang peptik dan kanser gastrik. Kaedah diagnostik invasif memerlukan prosedur endoskopik yang rumit bagi pengesanan *H. pylori*. Kaedah diagnostik tidak invasif sebaiknya boleh digunakan untuk mengurangkan tekanan kepada pesakit yang disyaki dijangkiti untuk menjalani prosedur pengesanan *H. pylori*. Terdapat dua kaedah diagnostik tidak invasif yang sering digunakan iaitu pengesanan antibodi (serologi) dan pengesanan antigen (pada najis) yang mempunyai kelebihan dan kekurangannya tersendiri. Urease adalah salah satu daripada antigen *H. pylori* yang mempunyai sifat imunogenik yang kuat. Oleh yang demikian, urease telah dipilih untuk pembangunan jalur ujian titik-EIA yang boleh digunakan dalam sistem pengesanan jangkitan *H. pylori* berasaskan serologi. Pengklonan fragmen urease A (pET32ureA3), fragmen urease B (pET32ureB2) dan keseluruhan operon urease (pET32UOA6) menghasilkan urease A rekombinan (UreA), urease B rekombinan (UreB) dan kompleks enzim urease rekombinan (UreA/UreB) yang aktif secara biologi setelah disahkan melalui esei berfungsi keimunan dengan menggunakan antibodi komersial urease- α *H. pylori* dan urease- β *H. pylori* dari Santa Cruz, Inc, USA. Penghasilan kompleks enzim urease rekombinan (UreA/UreB) menunjukkan satu operon urease atau replikon urease yang berfungsi telah berjaya dihasilkan. Penulenan urease rekombinan telah berjaya dilakukan dan urease rekombinan yang tulen masih aktif secara biologi. Urease rekombinan telah disalut ke membran sebagai persediaan untuk jalur ujian titik-EIA. Jalur ujian titik-EIA yang tersedia

memberi titik berwarna perang untuk menunjukkan tindak balas positif apabila dikesan menggunakan antibodi komersial terhadap urease (Santa Cruz, Inc, USA). Sembilan ekor arnab telah digunakan untuk menilai sifat keimunogen urease. Sera dari haiwan yang telah dicabar memberikan pengesanan positif di atas jalur ujian yang tersedia, seperti pengesanan menggunakan antibodi komersial, menunjukkan urease rekombinan mampu bertindak sebagai imunogen seperti urease asli. Tiga kumpulan mengandungi tiga ekor arnab setiap satu telah dicabar masing-masing dengan UreA rekombinan dituliskan, UreB rekombinan dituliskan atau UreA/UreB rekombinan dituliskan telah menunjukkan kehadiran antibodi terhadap urease di dalam sera arnab tersebut di atas jalur ujian titik-EIA. Seekor arnab yang bertindak sebagai kawalan negatif, telah dicabar menggunakan serum albumin lembu (BSA), tidak menunjukkan tindak balas positif di atas jalur ujian titik-EIA. Kesimpulannya, kajian ini telah berjaya mencapai kesemua objektif: pengklonan, pengekspresan, penulenan urease rekombinan yang berfungsi disamping pembangunan jalur ujian titik-EIA. Jalur ujian titik-EIA ini menyediakan asas kepada pembangunan seterusnya esei enzim-berkaitan berasaskan serologi untuk tujuan pemeriksaan awal menggunakan urease dengan hasrat pembangunan esei yang mesra, mudah, murah dan pantas. Disamping itu, replikon urease *H. pylori* telah membuka peluang untuk pembangunan model haiwan termodifikasi genetik untuk tujuan kajian patogenesis *H. pylori*.

**PRODUCTION OF RECOMBINANT UREASE
FOR SCREENING OF *Helicobacter pylori* INFECTION**

ABSTRACT

Helicobacter pylori establishes infection inside human stomach lining and causing duodenal diseases, such as peptic ulcer and potentially into gastric cancer. The invasive diagnostic methods require unpleasant endoscopic procedure for *H. pylori* detection. Preferably, the noninvasive diagnostic methods would make suspected patients less stressful to procedure for *H. pylori* detection. The two most preferable noninvasive diagnostic methods are antibody detection (serology) and antigen detection (from fecal) with their own advantages and drawbacks. *H. pylori* urease is one of the antigens found in *H. pylori* with strong immunogenic property. Thus, urease was chosen for the development of *H. pylori* dot-EIA test strip which could be used in a serology based detection system for *H. pylori* infection. Cloning of urease A gene fragment (pET32ureA3), urease B gene fragment (pET32ureB2) and the whole of urease operon (pET32UOA6) produced biologically active recombinant urease A (UreA), recombinant urease B (UreB) and recombinant urease enzyme complex (UreA/UreB) verified by immune functioning assay using commercial antibody *H. pylori* urease- α and commercial antibody *H. pylori* urease- β from Santa Cruz, Inc, USA. The production of recombinant UreA/UreB complex indicates that a fully functional urease operon or a urease replicon was successfully constructed. Purifications of the recombinant ureases were successful and the purified recombinant ureases were still biologically active. The purified recombinant ureases were coated onto membranes in preparation of dot-EIA test strips. The prepared dot-EIA test strips gave brown colour dots indicating positive reactions when probed

with commercial antibodies against ureases (Santa Cruz, Inc, USA). Nine rabbits were used to assess the immunogenicity properties of the recombinant ureases. Sera from the challenged animals gave positive detections on the prepared test strips, similar to detections using commercial antibodies, indicating the recombinant ureases could act as immunogens comparable to native ureases. Three groups containing three rabbits each were challenged with purified recombinant UreA, UreB or UreA/UreB respectively showed the presence of ureases antibodies in their sera on dot-EIA test strips. One rabbit that served as a negative control, challenged with bovine serum albumin (BSA), did not give positive reaction on dot-EIA test strip. To conclude, this study was successfully achieving all the objectives: cloning, expression and purification of functioning recombinant ureases, as well as, developing dot-EIA test strip. With the intention to develop user friendly, easy, cheap and fast detection; this dot-EIA test strip provides a foundation for further urease enzyme-linked serology based assay development as a mean for early screening. In addition, the constructed *H. pylori* urease replicon opened an opportunity for developing a genetically modified animal model to study *H. pylori* pathogenesis.

1.0 INTRODUCTION

Helicobacter pylori is one of the common bacterial infections in human and recognized as the etiologic agent for majority of upper gastro duodenal diseases. *H. pylori* has been established as the causative agent for acute or chronic gastritis (Mitchell, 1999) and could be further developed into peptic ulcer disease, gastric carcinoma and others upper gastro duodenal diseases (Kiesslich *et al.*, 2005, Ardekani *et al.*, 2013). According to the World Health Organization (WHO) statistic, *H. pylori* infection is on the rise and proportional to the progress of a country. Almost 50% of the world's population is infected by *H. pylori* (Sasidharan *et al.*, 2008). In Malaysia, *H. pylori* infection is on the raise as well. From the year 2000 until 2007, patients infected by *H. pylori* were 30.4% of the gastro duodenal cases reported (Sasidharan *et al.*, 2008).

Urease is one of the pathogenic factors that help *H. pylori* colonizes the epithelium in the acidic environment of the stomach (Ardekani *et al.*, 2013). *H. pylori* urease displays enzyme-independent effects in mammalian models, mostly through lipoxygenases-mediated pathway (Uberty *et al.*, 2013). The urease would induce edema, neutrophil chemotaxis and shows apoptosis inhibition reverted in the presence of the lipoxygenase inhibitors esculetin (Uberty *et al.*, 2013).

In addition to its involvement in the pathogenesis process of *H. pylori* infection, urease is also a target for vaccination development (Volland *et al.*, 2006) besides being a suitable marker to use as a target protein for detecting presence of *H. pylori* infection among suspected gastrointestinal patient.

Diagnosis of *H. pylori* infection could be very unpleasant procedure to the patients, as well as, to the physicians that have to perform it. The frequently use method in diagnosis of *H. pylori* infection would involve endoscopic procedure for

tissue biopsy sample collection before rapid urease test (RUT), Polymerase Chain Reaction (PCR) and histology methods could be carried out (Goh, 1997). This invasive endoscopic procedure has to be carried without anesthetic and the use of long endoscope pushing down the patient's throat. Gagging and vomiting are quite common during the procedure. The patients have no choice and have to endure the procedure. This endoscopic procedure also gives a little bit stress to the physicians when looking at the patients' reactions. There are many occasions where after enduring this unpleasant endoscopic procedure, negative results were observed indicating the absence of *H. pylori* infection.

An enzyme-linked serology based assay is used to detect the presence of antibody or antigen in a given sample. In this type of assay, one of the components (either the antibody or antigen) would be immobilized onto a test strip and an enzyme-linked secondary antibody would be used to detect the formed antigen-antibody complex on the test strip (Ricci *et al.* 2007). The use of enzyme-linked assay to detect *H. pylori* infection shows test's accuracy similar to invasive urea breath test (UBT) (Vaira *et al.* (1999, 2000). Additionally, the enzyme-linked assay is capable to detect all the immunoglobulin isotypes (Vaira *et al.*, 2007). The amount of sample need for this kind of assay is just a small amount of blood and the result could be known as fast as in minutes with sensitivity and specificity of more than 92% (Gatta *et al.*, 2004).

It would be more beneficial and convenience to the patients, as well as, to the physicians if there is a non-invasive, quick and simple diagnostic method to screen for *H. pylori* infection. Currently, such diagnostic method is limited and expensive. In this study, a non-invasive dot-EIA test strip of enzyme-linked serology assay was developed to screen the presence of *H. pylori* antibody. This dot-EIA strip would

use only a few drops of blood and simple procedure to detect the presence of *H. pylori* antibody. The dot-EIA test strip could be used for the benefits of patient's pre-test screening of *H. pylori* infection.

1.1 Objective of the study

The specific objectives of this study were:

1. To construct recombinant clones carrying *H. pylori* urease genes.
2. To determine the immune functioning of the expressed recombinant ureases.
3. To purify the recombinant ureases.
4. To develop a dot-EIA test strip to be used for screening of *H. pylori* infection.

2.0 LITERATURE REVIEW

2.1 Brief history of *H. pylori* discovery

The existence of spiral-shaped bacteria in human intestine has been known since early 19th century (Goodwin *et al.*, 1989). On 14 April 1984, Marshall & Warren who served in the Department of Microbiology at Royal Perth Hospital, Western Australia claimed that the presence of spiral-shaped organisms were colonizing the human intestine and eventually succeeded in culturing them. These bacteria were initially named as *Campylobacter pyloridis*, and then renamed as *Campylobacter pylori* (Goodwin *et al.*, 1989). Further research completed in October 1989 revealed that *C. pylori* was not suitable to be classified under the genus *Campylobacter*. Hence, a new genera was introduced, namely *Helicobacter*. At the end, these spiral-shaped bacteria were once again renamed as *Helicobacter pylori* (*H. pylori*), replacing the former *C. pylori* (Goodwin & Armstrong, 1990).

The earliest research on human-infected by *H. pylori* was carry out histologically and ultra structurally without any *in vitro* cultivation. Steer & Newell (1985) were the very first scientists that had observed the existence of spiral-shaped bacteria in the gastric mucous-secreting cells. Despite the failure in cultivating these cells, they managed to prove its existence in the gastric epithelial cell under the electron microscope.

The complete genome of *H. pylori* was successfully sequenced with a total number of 1,590 genes were recorded (Tomb *et al.*, 1997). The complete genome information has become a great advantage in comparing *H. pylori* genome with the genomes of other pathogens that causing upper gastro duodenal diseases in humans (Schlessinger, 1995).

2.2 Classification of *H. pylori*

Classification based on classical phenotype associated with gastric inflammation (gastritis) has characterized *Helicobacter* species from various mammals (Vandamme, *et al.*, 1991; Versalovic & Fox, 2001). In general, gastric *Helicobacter* are spiral shape bacteria with monopolar or bipolar flagella (Fawcett *et al.*, 1999). Fatty acid analyses further divide gastric *Helicobacter* into two groups: the “*H. pylori*-*H. felis*-*H. heilmanni*” and the “*H. mustalae*-*H. suncus*”. Comparative analysis of 16S rRNA sequence put helicobacters into rRNA superfamily VI together with *Campylobacter* (rRNA cluster I), *Arcobacter* (rRNA cluster II) and *Helicobacter* (rRNA cluster III) (Vandamme *et al.*, 1991; Versalovic & Fox, 1999; Baltrus *et al.*, 2009).

Most gastric *Helicobacter* are clustered in “*H. pylori*-*H. felis*-*H. heilmanni*” group by phylogenetic analyses based on comparative 16S rRNA (Goodwin *et al.*, 1989). In contrast, “*H. mustalae*-*H. suncus*” group, such as, *H. mustalea* and the enteric avian species, *H. pametensis* (Dewhirst *et al.*, 1994), are quite different by phylogenetic analysis.

Based on these classification methods, *Helicobacter* belongs to Proteobacteria phylum, class of Epsilonproteobacteria, order of Campylobacterales and in the family of Helicobacteraceae (Versalovic & Fox, 2001). Figure 2.1 shows the relatedness among *Helicobacter*.

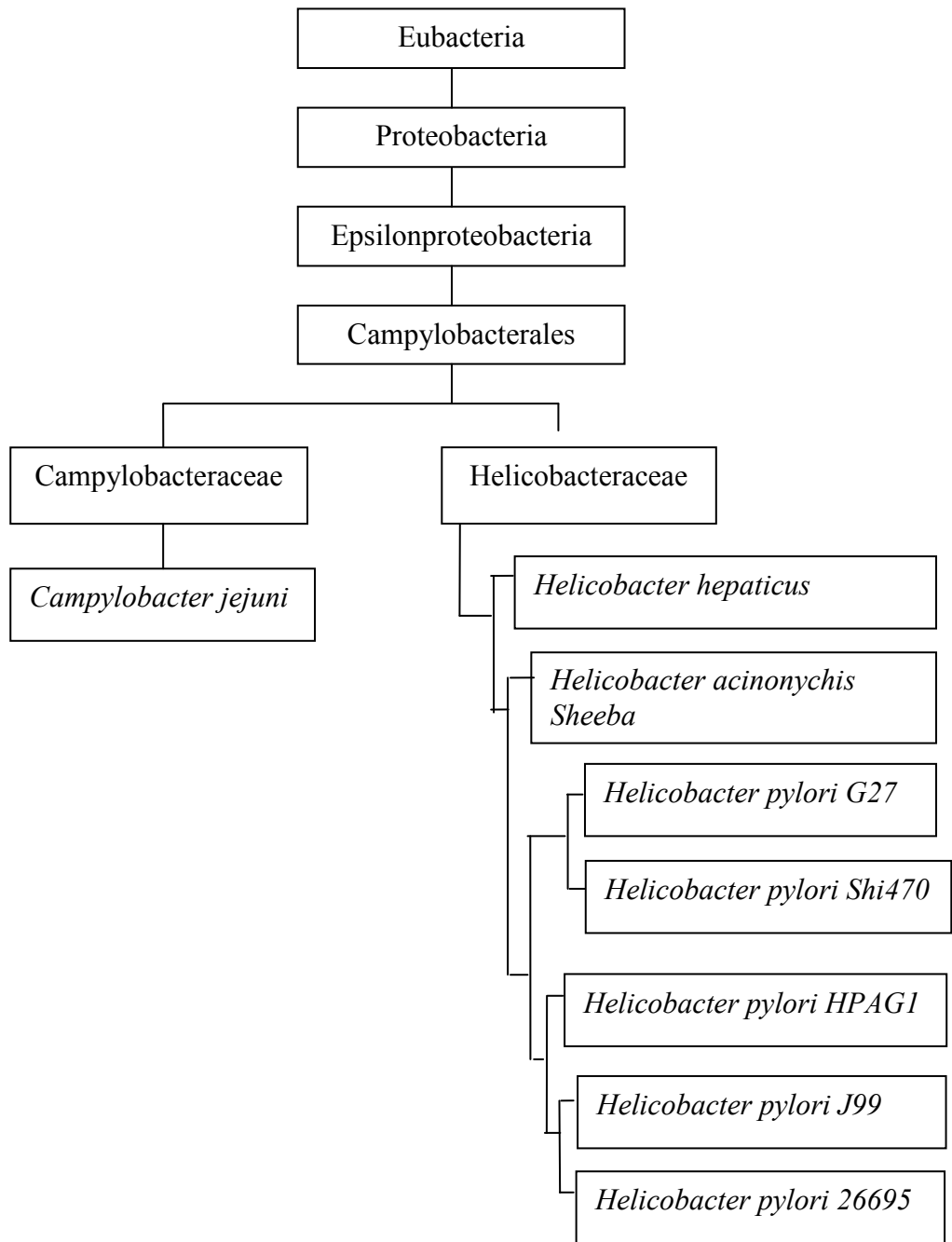


Figure 2.1: Relatedness of *Helicobacter* sp. (Versalovic & Fox, 1999; Baltrus *et al.*, 2009).

2.3 Phenotypic characteristics of *H. pylori*

Helicobacter pylori is a Gram-negative bacteria, spiral or curved bacilli and motile organism. It demonstrates itself as a spiral-shaped bacterium when it is in the culturable state (Goodwin & Armstrong, 1990). The length of this bacterium is in the range of 2.5 to 3.5 μm with a diameter ranging from 0.5 to 1.0 μm . *H. pylori* has a smooth surface with a capsule-like outer membrane. *H. pylori* is a lophotrichous bacterium that use the flagella to drive the bacterium in one direction (Abdulqawi *et al.*, 2012). *Helicobacter pylori* possesses two different morphologies which are spirally-shaped or curved and coccoid shape. *Helicobacter pylori* cells are spirally shape in a culturable young stage. However, the bacterium would become coccoidal shape during a mature state or prolonged culture. Figure 2.2 shows a mixed of spiral and coccoid shapes of *H. pylori* cells in the culture plate (Bode *et al.*, 1993).

Kuster *et al.* (2006) report that this bacterium would change its spiral-shape to coccoid shape when continuously exposed to oxygen or in the presence of bactericide. The coccoid shape is the dormant unculture stage that could be used as a confirmation for the existence of *H. pylori* (Andersen & Wadstrom, 2001). This condition is not only common in *H. pylori* but also in wide varieties of enterogenic pathogens (Wang & Wang, 2004). Other variables that induce *H. pylori* morphological changes would include aerobiosis, alkaline pH, high temperature, prolonged incubation period, proton-pump inhibitor and antibiotic treatments (Kusters *et al.*, 1997; Costa *et al.*, 1999; Wang & Wang, 2004).

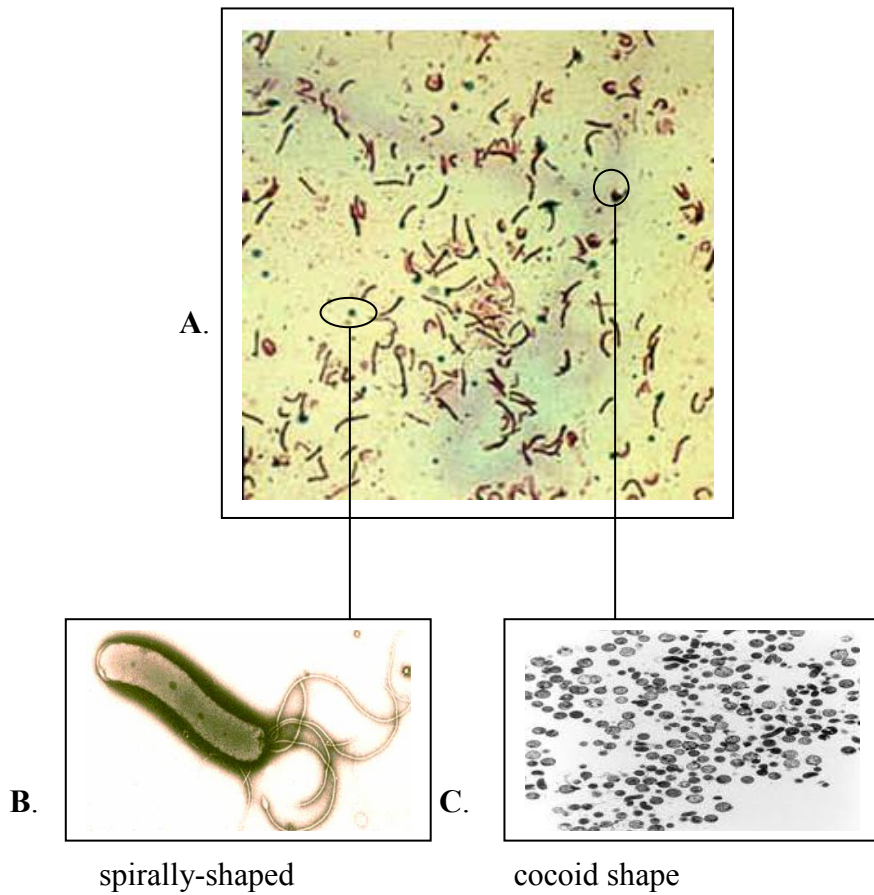


Figure 2.2: Two different morphologies of *H. pylori*. (A) Light microscope at 100X magnification (Bode *et. al.*, 1993), (B) SEM of spirally-shaped bacterium (<http://www.ivdbiocare.com/hp.htmlat>, access on 13/1/2014) and (C) SEM of cocoid shape bacterium (http://cfpub.epa.gov/ncer_abstracts/index.cfm/fuseaction/display.abstractDetail/abstract/823/report/E, access on 13/1/2014).

The ability of *H. pylori* to change its shape to coccoid while colonizing is one of the survival means which enables it to adapt to sudden changes in the environments (Andersen & Wadstrom, 2001). For patients that are under prolonged antibiotic treatments, *H. pylori* would transform itself to the coccoid shape and would revert back to spirally-shaped once the antibiotic treatment is stopped. This condition is not a re-infection but it is simply an activation from coccoid to spiral (Kusters *et al.*, 1997). The change from coccoid to spiral does not affect *H. pylori* ability to produce ureases (enzymes responsible for *H. pylori* pathogenicity) and would result in the damage to the mucoid tissue of the gastrointestinal lining (Andersen *et al.*, 1997; Tominaga *et al.*, 2001).

Helicobacter pylori possess five major outer membrane protein (OMP) families (Kusters *et al.*, 2006). The largest family would include putative adhesions and the other four families are porins, iron transporters, flagellum-associated proteins and proteins of unknown function. The outer membrane of *H. pylori* consists of phospholipids and lipopolysaccharide (LPS). The O antigens of LPS may be fucosylated (the process of adding fucose sugar units to a molecule) and mimic Lewis blood group antigens and also contains cholesterol glucosides (Kusters *et al.*, 2006).

2.4 *Helicobacter pylori* cultivation

The morphological changes of *H. pylori* from spiral to coccoid cause the cultivation and preservation techniques to be essential. In order to ensure that the culture stay fresh, the subculturing of *H. pylori* onto fresh medium and its maintenance has been extensively emphasized (Andersen & Wadstrom, 2001)

In the laboratory, *H. pylori* is cultured according to Goodwin & Armstrong (1990) and Tompkins (1992), where agar containing 10% blood is used. The culture would be incubated in a microaerophilic incubator with 100% humidity and mixture of air with 10% CO₂. The function of the blood in the medium is to absorb toxic materials, such as, free oxygen radicals which are highly poisonous (Tompkins, 1992). The high humidity inside the incubator is to ensure the growth of the bacteria (Goodwin *et al.*, 1989). *Helicobacter pylori* is considered as a slow-grower and its colonies are normally visible after four to seven days of incubation. The size of the *H. pylori* colony is in the range of 1 to 2 mm in diameter with transparent appearance. In addition, hemolysis on the blood agar could be seen for spirally shape *H. pylori* in 84 hours (Goodwin *et al.*, 1989).

Helicobacter pylori's colonies would be transformed into cocoid after seven days of incubation (Catrenich & Makin, 1991). This morphological alteration is induced by the accumulation of ammonia which influence the environmental pH of the medium. A continuous exposure to oxygen would kill the bacteria. In order to ensure its viability, subculturing of *H. pylori* onto fresh medium has to be done in every three to four days (Tompkin, 1992).

Enzyme assay, such as, urease, oxidase, catalase, alkaline phosphatase, glutamyl transferase and various esterase (C₄ – C₁₂) could be used to confirm *H. pylori* culture (Megraud *et al.*, 1985). *Helicobacter pylori* produces a large amount of ureases compared to *Ureaplasma urealyticum* and for that becomes the most common enzyme assay for *H. pylori*.

Helicobacter pylori could be cultured on various commercial solid media such as Eugon agar, Mueller Hinton agar, egg yolk emulsion agar, Skirrow agar, Dent agar, Thayer-Martin agar, soy trypticase agar and brusela agar (Hartzen *et al.*,

1997; Piccolomini *et al.*, 1997). Other than that, liquid culture is another alternative to cultivate this bacterium. Such broth would be eugon, soy trypticase, brusela, Columbia and Mueller-Hinton (Shahamat *et al.*, 1991). The final ingredient is blood that would be added to a final concentration of 10%.

2.5 Virulence Factors of *H. pylori*

Virulence is defined as the ability of an infectious agent to produce disease. The virulence of a microorganism is a measure of the severity of the disease it causes (Elizabeth, 2010). The *H. pylori* has several virulence factors that contribute to gastric colonization, tissue damage and survival factors in human gastric mucosa as shown in figure 2.3. Such factors would include ureases which produce ammonia, flagella and adhesions factor (Andersen & Wadstrom, 2001). *Helicobacter pylori* also secretes exotoxins, such as, cytotoxin, protease, catalase, phospholipase A2 and platelet-activating factor (PAF) (Wyle *et al.*, 1993).

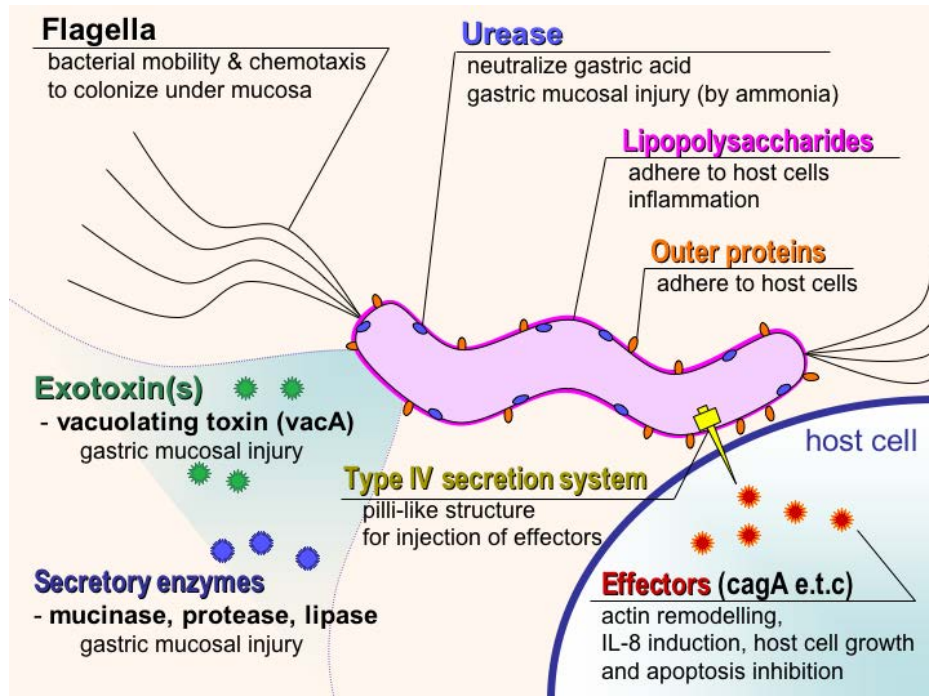


Figure 2.3: Virulent factor in *H. pylori*
(http://commons.wikimedia.org/wiki/File:H_pylori_virulence_factors_en.png
, access on 15 January 2014)

2.5.1 Flagela

Flagellum is an important virulence factor for *H. pylori* as it functions primarily in the locomotion of cells, especially for the onset of continuous colonization at the gastric mucosa (Eaton *et al.*, 1996). The gene that is responsible in the expression of flagellum is *flaA* (Vandenplas, 2000). Normally, *H. pylori* possesses two to six flagella at the end of one pole which are joined to the outer membrane where it consists equally of lipopolysaccharide, phospholipid and protein (Geis *et al.*, 1993). The flagellum was divided into two subunits, which were encoded by the *flaA* and *flaB* genes, respectively (Josenhans *et al.*, 1995).

The flagellum enables *H. pylori* to swim and move in the lumen of the stomach of its host before attaching to the epithelium cells and the mucoid layer of the stomach (Eaton *et al.*, 1992). The movement that is created by the flagellum enables *H. pylori* to enter neutral areas of the stomach layer, such as the epithelium layer and underneath the mucosa layer in order to avoid the epithelium surface area that is highly acidic. Josenhans *et al.* (1995) had also reported that mutagenesis at both *flaA* and *flaB* genes had further deduced that the flagellum acts primarily as an organelle of locomotion which help in the colonization in *gnotobiotic* piglets (Eaton *et al.*, 1996).

2.5.2 Urease

Urease is a crucial virulence factor for *H. pylori* to initiate an infection. Urease is normally found at intracellular and extracellular of *H. pylori* cells (Eaton *et al.*, 1996). Urease has three subunits, UreA, UreB and UreC, encoded by the *ureA*, *ureB* and *ureC* genes, respectively. In addition, *H. pylori* has an accessory urease gene, *ureIEFGH*, to maintain a neutral-pH microenvironment around the bacteria

and necessary for survival in the acidic condition (Perez-perez *et al.*, 1992). The enzymes produced by *H. pylori* are mostly metabolic, antioxidant and toxic enzymes (Nilius & Malfertheiner, 1996). The acidity increment in the lining of human mucoid cells is important mechanism in the colonization process of *H. pylori*. Urease is a cytoplasmic enzyme (Phadnies *et al.*, 1996) that hydrolyzes urea into bicarbonate and ammonia which eventually contributes to the increment of environmental pH value. Ammonia is a source of nutrient for bacteria, as well. In doing so, it also harms the gastric epithelial cells (Figura, 1997).

Urease (Urea amidohidrolase, EC 3.5.1.5) catalyzes the hydrolysis reaction of urea to produce ammonia and carbamate. Carbamate then undergoes spontaneous hydrolysis to yield ammonium and carbonic acid. The former would continue to react with water molecules to form ammonium hydroxide which leads to the increase of pH. The urease activity of *H. pylori* produces a strong base such as ammonia and bicarbonate that neutralizes acidic environment of the stomach's acids. As a result, *H. pylori* is free from the danger of the stomach's acids before attaching to the epithelium layer of the human stomach (Eaton *et al.*, 1991). At the outer surface of the mucus layer, there is a sulfhydryl group that functions to protect endogens. However, the ammonia that was produced from the urease reaction could lead to the rapid reduction of glutenin, an example of the sulfhydryl group (Nagy *et al.*, 1990). This situation aggravates the damage of the mucoid tissue in the stomach.

2.5.3 Type IV secretion system

The attachment mechanism of *H. pylori* to the gastric epithelial cells is the most crucial factor for the colonization of *H. pylori* in the human stomach. An attachment site and receptor site are needed for the attachment of *H. pylori* to occur (Dubreuil *et al.*, 2002). The attachment sites on bacteria would include proteins, glycoconjugates or lipids which become the initial sites of colonization after *H. pylori* infection. While the receptor sites on the surface of human epithelium cell could be lipid, protein, glycolipid or glycoprotein (Castillo-Rojas *et al.*, 2004). The attachment mechanism of *H. pylori* to the gastric epithelial cells has yet to be completely comprehended.

However, it was understood that *H. pylori* produces attachment substance which is injects at least one modulating actor directly into the host cell cytosol, the product of the cytotoxin associated gene A (*cagA*) that is related to type IV secretion system that enables it to adhere to the lipid-related membrane and carbohydrate (Gupta, 2009). This kind of substance strengthens the attachment of *H. pylori* to the mucoid epithelial layer and prevents it from being easily washed off. Moreover, there are also several attachment sites that are involved in the attachment process to the surface of epithelial cells for colonization to occur (Wadstrom *et al.*, 1993). *Helicobacter pylori* is capable of colonizing a few locations which includes in the mucus, adhere to the epithelial cells and the lumen of the epithelial cells (Marshall *et al.*, 2002).

2.5.4 Secretory enzymes

Cysteine protease is a proteolysis enzyme which also could cause damage to the stomach tissues and cells. The presence of ammonia as a result of the *H. pylori*'s activity ables to induce the excretion of cysteine protease into the stomach (Szabo *et al.*, 1992). Due to the reasons where cysteine or thiol protease (for example: cathepsin B, H and L) are involved in the catalyzation of intracellular and extracellular digestion, cysteine protease is capable of destroying and damaging the gastric mucus (Szabo *et al.*, 1992).

2.5.5 Exotoxin

Exotoxin is defined as a potent toxin formed and excreted by the bacterial cells to the surrounding medium (Elizabert, 2010). One of the most important virulence factors, the vacuolating cytotoxin (VacA), which is the only known exotoxin released by *H. pylori* into the stomach of infected individuals, contributing to the pathogenesis of infection. The important virulence factor of *H. pylori* is the vacuolating cytotoxin (VacA), encoded by the *vacA* gene.

VacA was initially named for its ability to induce the formation of large intracellular vacuoles within the cytoplasm of cultured mammalian cells and more recently has been directly implicated in the epithelial erosion preceding the formation of both gastric and duodenal ulcers (Gupta, 2009). However, this epithelial cell vacuolization, forms channels in epithelial cell membranes also providing the bacterium with nutrients (Oona, 2005). In the early discovery of *H. pylori*, it was revealed that this bacteria excreted cytotoxins (exotoxin) that cause the formation of cytoplasmic vacuolation in eukaryotic cells. The isolation of *H. pylori* from ulcer

patients has demonstrated such condition and this has suggested that the cytotoxins are able to react in the formation of peptic ulcer (Figura *et al.*, 1989).

Helicobacter pylori stimulates the excretion of pepsinogen, a substance that is necessary in the production of pepsin (one of exotoxin type) in the human stomach. Pepsin is a type of proteolysis enzyme that damages mucus cells and it has the potentials to act as an aggressive factor in the development of ulcer in the duodenum. This explains how *H. pylori* causes ulcer in duodenum in patients that are infected (Trust *et al.*, 1991).

Helicobacter pylori produce another exotoxin, phospholipase (PL) and this element disrupts the structure of cell membrane and eventually destroys it. PL reacts directly with lecithin in order to produce lysolecithin which acts as cytotoxin and this cytotoxin would destroy the cell membrane. Due to the colonization of *H. pylori* in the stomach's mucus and the present of small amount of PL is sufficient in bringing extensive damages to the mucous cell membrane (Weitkamp *et al.*, 1993). A study by Rhodes *et al.* (1996) had proven that the levels of lysolecithin in gastric juice of patients with ulcer were relatively higher compared to normal individuals.

Further, *H. pylori* also could produce another exotoxin, phospholipase A2 and C after their colonization in the human stomach which involve in the inflammation and mucosal damage associated with peptic ulcer formation (Daw *et al.*, 1990, Langton & Cesareo, 1992).

2.5.6 Lipopolysaccharide

The outer membrane cell of *H. pylori* contains lipopolysaccharide (LPS) component. This LPS component is *H. pylori*'s antigen and endotoxin (Slomiany *et al.*, 1991). The LPS acts as adherence ligands for *H. pylori* to the adhesion structure of sialyllactose on human gastric epithelial cells. After infection of *H. pylori* to the epithelial cells of the gastric antrum, the LPS were expressed and destroyed the integrity of stomach cells. This response would increase the inflammation and injury to human gastric epithelial cells (Slomiany *et al.*, 1991; Trust *et al.*, 1991).

2.5.7 Effectors

Helicobacter pylori also inject at least one modulating factor directly into the host cell cytosol, the product of the cytotoxin associated gene A (*cagA*) (Gupta, 2009). The *cag* PAI protein is another crucial virulence factor in *H. pylori* infection. It is known as immuno-dominant antigens which is located at the cells surface of the bacteria. This protein is present in 50–60% of strains of *H. pylori* (Tummuru *et al.*, 1993; Solca *et al.*, 2000). The production of *cag* PAI protein by *H. pylori* strain is highly correlated with the peptic ulcer disease and several other gastric diseases (Tummuru *et al.*, 1993; Censini *et al.*, 1996). Translocated CagA protein was activates NF-kB (type of effector) leading to a cascade effect, resulting in the release of inflammatory mediators such as interleukin-8 (IL-8) (Gupta, 2009). This IL-8 was captivating inflammatory cells to accumulate at the point of infection by *H. pylori* (Covacci *et al.*, 1993).

The explanation of *H. pylori* immuno-inflammation to human gastric still lacking but there were hypotheses made which relate gastritis to human gastric inflammation. The hypotheses were relating gastritis with the effector effect of

excretion for leukocyte chemotaxis factor by *H. pylori*. These hypotheses were then proved with the discovery of *H. pylori* producing chemotaxis factor that attracted monocytes and neutrophil towards the cells in the growth medium (Craig *et al.*, 1992). Other researcher also reported that chemotaxis factor of *H. pylori* was permeate into the mucus layer and activate the immune system and leukocytes of human gastric (Wallace *et al.*, 1990). At the same time, the endothelium cells in the human stomach secreted interleukin-8 (IL-8) (Strieter *et al.*, 1992), which was a strong chemotaxis factor. Both of these factors resulted in the chemical attraction toward polymorph nuclear cells, such as, leukocyte and neutrophil. Leukocyte and neutrophil would excrete free oxygen radicals and proteolysis enzymes that harmful to the cells. These free oxygen radicals and proteolysis enzymes would be accumulate on mucus layer of human gastric and would be continuous express until the onset of chronic gastritis and ulcer became more serious (Strieter *et al.*, 1992).

Denizot *et al.*, (1990) also reported that *H. pylori* was able to synthesize another effectors, platelet-activating factor (PAF) when it was cultured in agar that contains blood but not in broth medium. PAF is a substance that causes inflammation on human tissues and it is usually produced by macrophage, neutrophil, endothelium cell and platelet. PAF induces chemotaxis effect towards leukocyte and would lead to the change in vascular permeability in human stomach (Denizot *et al.*, 1990).

2.5.8 Outer protein

Helicobacter pylori outer membrane proteins (OMPs) are essential for metabolism while maintaining the selective permeability (O'Toole & Clyne, 2001). The *H. pylori* OMPs have five porins that are identified and labelled as HopA through E based on their functionalities characterization (Exner *et al.*, 1995).

The porin-OMPs of *H. pylori* are bound to carbohydrate ligands on epithelial cells (Exner *et al.*, 1995). The BabA and the SabA adhesins belong to a large family of *H. pylori* outer-membrane proteins (Hop). The receptor of the blood group antigen-binding adhesin BabA which binds Lewis b antigen (Le^b) related to ABO blood group antigens (Aspholm-Hurtig *et al.*, 2004). Furthermore, the receptors of sialic acid-binding adhesion, SabA, are bound to sialyl-Lewis x/a antigens of *H. pylori* (Mahdawi *et al.*, 2002). The ability of many *H. pylori* strains to adhere to sialylated glycoconjugates expressed during chronic inflammation might thus contribute to virulence of *H. pylori* infection to human (Bäckström *et al.*, 2004).

2.6 Transmission of *H. pylori* infection

Helicobacter pylori infection may occur by direct or indirect transmission. The direct transmissions could be in the forms of oral-oral, gastro-oral or faecal-oral (Khalifa *et al.*, 2010). The human stomach is the main reservoir for *H. pylori* and the bacteria could be transferred from human stomach to the external environment by vomiting and faeces (Oona, 2005). The indirect transmission would be the route for *H. pylori* infection back into human stomach through contaminated water and food sources, as well as, environmental conditions (Khalifa *et al.*, 2010).

2.6.1 Oral-Oral Transmission

Studies by Li *et al.*, (1996), Kim *et al.*, (2000) and Song *et al.* (2000b) reported the existence of *H. pylori* in human mouth. High prevalence of *H. pylori* acquisition was found in the saliva and dental plaque. All of these findings suggested the presence of *H. pylori* in the human mouth and its transmission could have occurred during food ingestion.

Mouth to mouth transmissions could have occurred in dental clinics as well. A study shows that the prevalence of *H. pylori* infection among dental nurses was higher than the dentists (Hazell *et al.*, 1987). This might be due to the amount of time spent by the dental nurses cleaning the teeth.

Similar prevalence could be observed among gastroenterologists where some of them might have overlooked hygiene practices after performing endoscopic examinations on patients. The possible acquisition of *H. pylori* infection is when the gastroenterologist's mouth comes too close in contact with the endoscope while biopsy samples are taken. For this reason, *H. pylori* infection is more prevalence among gastroenterologists compared to nurses and assistant medical officers at Kuala Lumpur Hospital (Goh *et al.*, 1998).

In Chinese culture, eating with chopsticks could be another possible route for mouth to mouth transmission (Leung, 1999). This could explain the high occurrence of *H. pylori* infection in the Chinese community compared to those that do not practice eating using chopsticks (Chow *et al.*, 1998). However, different trends of seroprevalance were observed among different Chinese communities where Malaysia and Singapore Chinese shows lower seroprevalance than those from China, Hong Kong, Vietnam and elsewhere as reported by Chow *et al.* (1995).

2.6.2 Gastro-oral Transmission

The gastro-oral transmission of *H. pylori* is associated with the transmission of *H. pylori* from saliva and gastric juices of infected patients to the health care personnel during endoscopy procedure (Pius *et al.*, 2000). Nevertheless, the prevalence of *H. pylori* transmission through gastro-oral transmission shows disparity among different countries. This disparity is further extended among the practitioners, such as, doctors, nurses health assistants and others (Pius *et al.*, 2000; Triantafillidis *et al.*, 2002; Mastromarino *et al.*, 2005; Mastromarino, 2006). Studies carried out in Germany and few other countries show no significant correlations between groups of health care staffs and the prevalence of *H. pylori* infection Mitchell *et al.*, 1989; Braden *et al.*, 1997; Pius *et al.*, 2000).

However, in Malaysia, there was increasing number of *H. pylori* infections among the doctors or nursing staff (Sasidharan, 2002). The *H. pylori* transmissions could have occurred due to disregard of safety measures, such as, not wearing gloves and face masks during the endoscopy procedure. This could lead to the accidental exposure and acquisition of *H. pylori* among the doctors and nursing staff. Similar situation was observed in Greece where higher prevalence of *H. pylori* infection via gastro-oral transmission among the health care personnel (Goh *et al.*, 1996; Mastromarino, 2006). Consequently, gastro-oral transmission of *H. pylori* is dependent on the adherence of the health care personnel with regards to safety and cleaning procedures. The use of special gloves has no guarantee of providing extensive protection to the endoscopists and supporting staff against *H. pylori* as the remaining drops of gastric juice on the endoscopic apparatus might have caused *H. pylori* to be spread more easily (Pius *et al.*, 2000). In order to eliminate the possibility of transmission through endoscopic apparatus, specifically designed fluid

repellent masks, eye protection, face shields and fluid resistant aprons or gowns should be worn when using the endoscopes and accessories. It is absolutely necessary to carry out proper cleaning and disinfection of the endoscopes and accessories before and after endoscopic procedure, to reduce and perhaps eliminate the possible *H. pylori* infection via gastro-oral transmission (Pius *et al.*, 2000).

2.6.3 Fecal-oral Transmission

Due to lack of proper hygiene practices in Chile, water sources were easily contaminated with *H. pylori*. The farmers, then, used this contaminated water sources to water their plants (Hopkins *et al.*, 1993). The fecal contaminations, now had the chances, to transfer to human when vegetable and fruit products were not washed properly before consumed. Therefore, this would cause higher chances of *H. pylori* infections in human. Thomas *et al.* (1993 & 1999) had successfully isolated *H. pylori* from 9 out of 20 stool samples collected from children under 2 years of age. Hence, this was another supportive evidence for the possibility of *H. pylori* infection via faecal oral route transmission if proper hygiene practices were lacking. Thus, good practice of proper sanitary hygiene and system, as well as, dietary habits have been known to play significant roles in limiting *H. pylori* transmission. It is also advisable to ensure raw foods are well-cooked before ingesting in order to reduce the prevalence of *H. pylori* transmission (Brown *et al.*, 2001).

2.6.4 Contaminated water, food and environmental conditions

Helicobacter pylori infection was found to be higher in developing countries compared to developed countries due to poor management of water drainage system and water resources that were contaminated with the bacteria (Klein *et al.*, 1991). The number of peptic ulcer patients could be reduced if good hygiene were practiced including boiled drinking water and water free from contaminated *H. pylori*. The risk factors related to the acquisition of *H. pylori* in infants and children at early ages included living in a low socioeconomic status area, over-crowded population and even the bed-sharing among different families (Vandenplas, 2000).

Segal *et al.* (1996) and Wadstrom *et al.* (1993) reported that water played an important role as a source of contamination of *H. pylori*. In fact, majority of *H. pylori* infections in humans are water related. *Helicobacter pylori* would remain as cocoid dormant stage in the contaminated water and would change into infectious spiral-shaped bacteria once inside human to start a new infection (Vale & Vítor, 2010). This infection would cause damage to the host's epithelial cells (Vale & Vítor, 2010).

In Peru, studies have been carried out on several external water resources by utilizing PCR technique and shown that few of the water resources caused increase chances of infection in the population by 12-fold (Hulten *et al.*, 1996). Apart from that, some other studies had also been conducted by using the ^{13}C urea breath test technique in order to investigate the correlation between the drinking water supply and the infection of *H. pylori* among children in Peru. The results of such studies had shown high correlations between *H. pylori* infection and socioeconomic status. The prevalence of infection was found to be higher among children from low-income families compared to those from high-income families (Klein *et al.*, 1991).