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_2O	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

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

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PENGHASILAN REKOMBINAN UREASE

UNTUK PENGESANAN JANGKITAN *Helicobacter pylori*

ABSTRAK

Helicobacter pylori mempunyai keupayaan 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 digunapakai 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

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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 ditulenkan, UreB rekombinan ditulenkan atau UreA/UreB rekombinan ditulenkan 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 tidak 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*.

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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

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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.

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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 (Uberti *et al*., 2013). The urease would induce edema, neutrophil chemotaxis and shows apoptosis inhibition reverted in the presence of the lipoxygenase inhibitors esculetin (Uberti *et al*., 2013).

In addition to its involvement in the pathogenesis process of *H. pylori* infection, urease is also a target for vaccination development (Voland *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

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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

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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.

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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 became a great advantage in comparing *H. pylori* genome with the genomes of other patogens that causing upper gastro duodenal diseases in humans (Schlessinger, 1995).

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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. heilmannii*" 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. heilmannii*" 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*.

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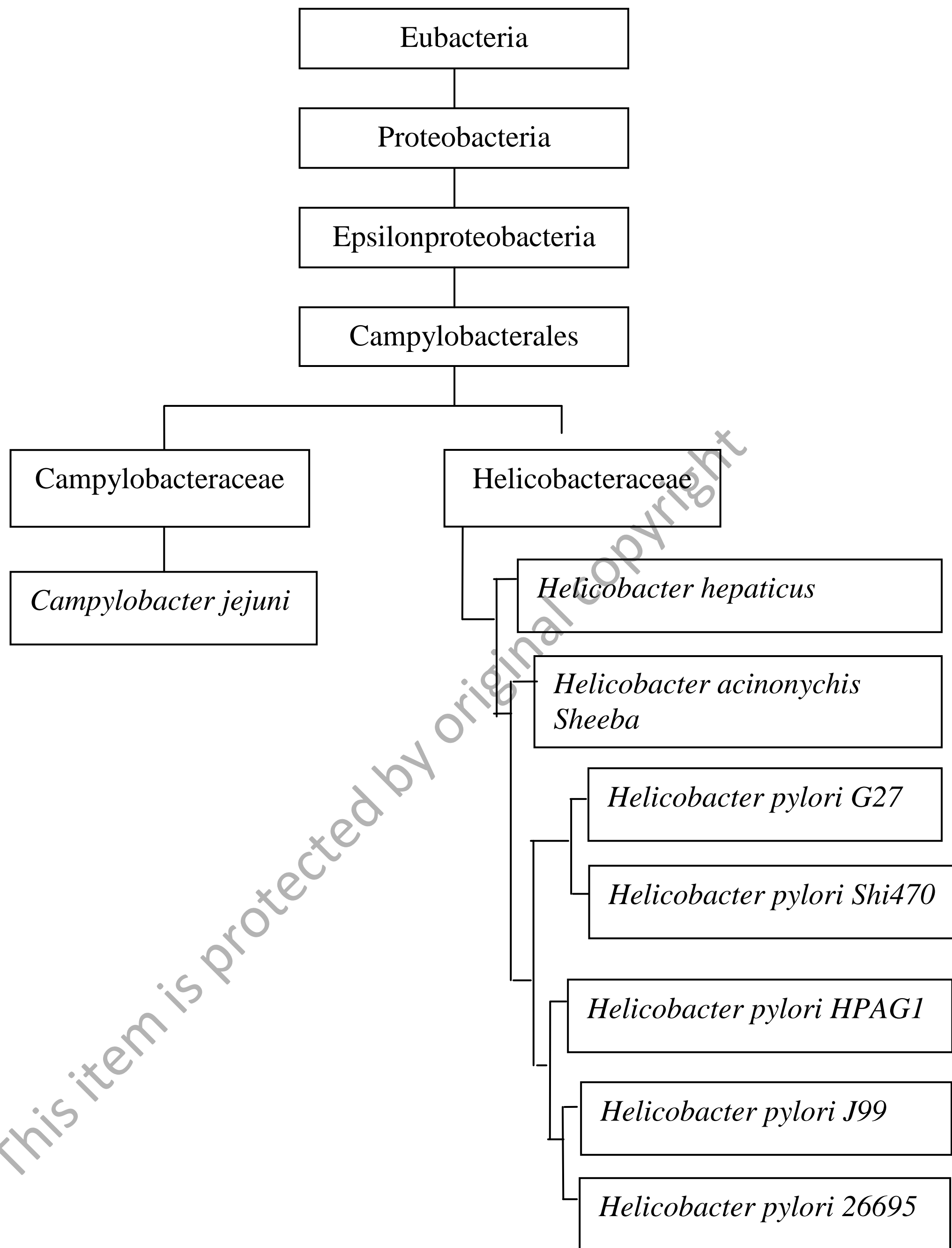

Figure 2.1: Relatedness of *Helicobacter sp.* (Versalovic & Fox, 1999; Baltrus *et al*., 2009).

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2.3 Phenotypic characteristics of *H. pylori*

Helicobacter pylori is a Gram-negative bacteria, spiral or curved bacili 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* posseses two different morphologies which are spirally-shaped or curved and cocoid shape. *Helicobacter pylori* cells are spirally shape in a culturable young stage. However, the bacterium would become cocoidal shape during a mature state or prolonged culture. Figure 2.2 shows a mixed of spiral and cocoid 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 cocoid shape when continuously exposed to oxygen or in the presence of bactericide. The cocoid shape is the dormant unculture stage that could be used as a confirmation for the existance 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).

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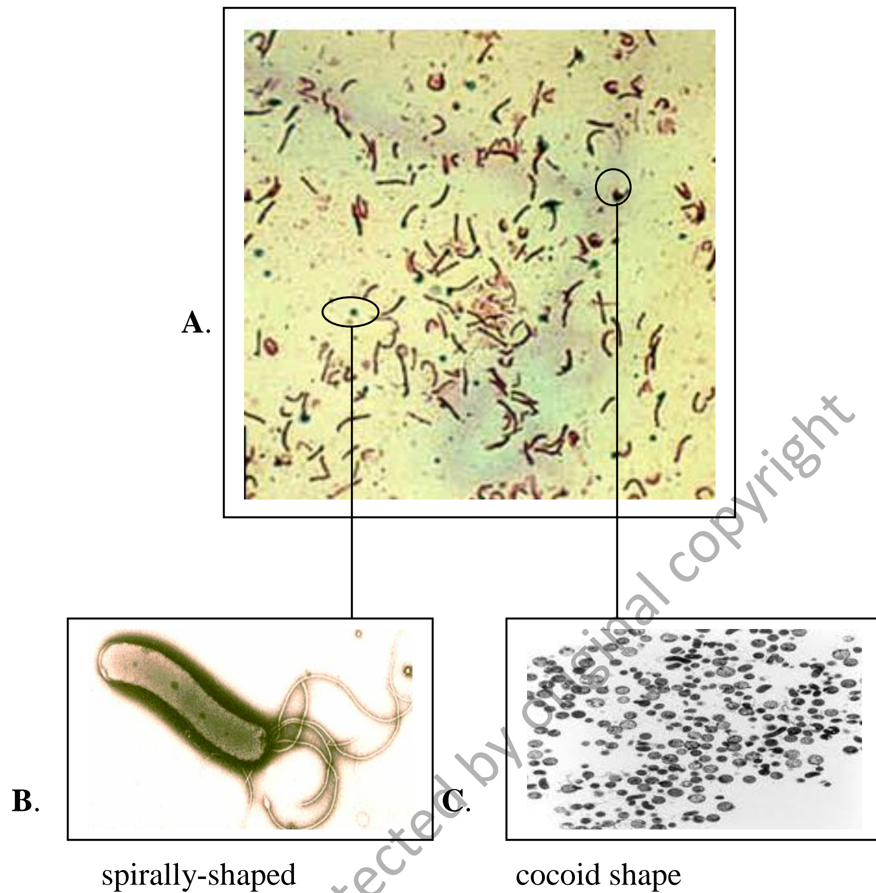

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Figure 2.2: Two different morphologies of *H. pylori.* (A) Light microscope at 100X magnification (Bode *et. al*., 1993), (B) SEM of spirally-shaped bacterium (http://www.ivdbiocare.com/hp.htmlat, access on 13/1/2014) and (C) SEM of cocoid shape bacterium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

The ability of *H. pylori* to change its shape to cocoid 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 cocoid 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 cocoid to spiral (Kusters *et al*., 1997). The change from cocoid to spiral does not affect *H. pylori* ability to produce ureases (enzymes responsible for *H. pylori* pathogenicity) and would resulted 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 fucosa sugar units to a molecule) and mimic Lewis blood group antigens and also contains cholesterol glucosides (Kusters *et al*., 2006).

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2.4 *Helicobacter pylori* cultivation

The morphological changes of *H. pylori* from spiral to cocoid 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% CO_2. 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 (C_4 – C_{12}) 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*.,

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