CLONING, EXPRESSION AND CHARACTERIZATION OF A NOVEL *HELICOBACTER PYLORI* DIFFERENTIATING ANTIGEN – HEAT SHOCK PROTEIN 20

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NATIONAL UNIVERSITY OF SINGAPORE

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LIST OF ABBREVIATIONS

AA (aa): amino acid

AGE: acid glycine extract

BHI: brain heart infusion

BSA: bovine serum albumin

CBA: chocolate agar plate

CFU: colony-forming unit

4-CN: 4-chloro-napthol

CP: cytoplasmic protein

CO-IP: co-immunoprecipitation

***D*:** nucleotide divergence

DAB: 3,3'-diaminobenzidine

3-D: three-dimensional

2-DE: two-dimensional gel electrophoresis

DNA: deoxyribonucleic acid

DTT: dithiothreitol

DU: duodenal ulcer

ECL: enhanced chemiluminacence

EDTA: ethylenediaminetetracetic acid

ELISA: enzyme-linked immunosorbent assay

EM: electron microscopy

FITC: fluorescein isothiocynate

GU: gastric ulcer

HPLC: high performance liquid chromatography

HRP: horseradish peroxidase

HSP: heat shock protein

HSP20: heat shock protein 20

HSP60: heat shock protein 60

IAA: iodoacetamide

IEF: iso-electric focusing

IL: interleukin

IPG: immobilized pH gradient

IS: insertion sequences

Ka: nonsynonymous nucleotide position

kDa: kilo Dalton

Km: kanamycin resistant gene

$\boldsymbol{K_S}$**:** synonymous nucleotide positions

LB: Luria-Bertani

LPS: lipopolysaccharides

LVER: low viscosity epoxy resin

MALDI-TOF MS: matrix-assisted laser desorption/ionization-time of flight mass Spectrometry

ML: maximum likelihood

MS: mass spectrometry

MW: molecular weight

NUD: non-ulcer dyspepsia

OD: optical density

OMP: outer membrane protein

OPD: O-phenylenediamine dihydrochloride

OR: odds ratio

ORF: open reading frame

PAI: pathogenicity island

PBS: phosphate buffered saline

PBST: phosphate buffered saline & Tween-20

PCR: polymerase chain reaction

PDB: protein database

PSB: phosphate saline buffer

PUD: peptic ulcer disease

PVDF: polyvinylidene difluoride

Q-TOF MS: quadrupole time of flight mass spectrometry

RAPD: randomly amplified polymorphic DNA

rCagA: recombinant CagA protein

rHSP20: recombinant heat shock protein 20

RNA: ribonucleic acid

RT-PCR: reverse-transcriptase polymerase chain reaction

SDS-PAGE: sodium dodecyl sulfate -polyacrylamide gel electrophoresis

SOD: superoxide dismutase

TAE: Tris-Acetate-EDTA

TE: tris-EDTA buffer

TEM: transmission electron microscopy

TP: total protein

WB: western blotting

LIST OF PUBLICATIONS

Research papers published:

1) Rui Juan Du & Bow Ho

Surface localized Heat Shock Protein 20 (HslV) of *Helicobacter pylori*

Helicobacter, 8(4), 2003, 257 – 267.

2) Rui Juan Du and Bow Ho

Heat Shock Protein 20 as a potential colonization factor and chaperon of CagA in *Helicobacter pylori* infection in mice

Submitted.

3) Rui Juan Du[1], Sook Yin Lui[1], Balbir Chaal[1], Khay Guan Yeoh[2], Douglas E. Berg[3], Nobuo Aoyama[4], Torkel Wadström[5] and Bow Ho[1]

Heat Shock Protein 20 of *Helicobacter pylori* – A novel epidemiological and gastroduodenal disease differentiating marker

Submitted.

Posters presented in the International Conference:

1) R. J. Du and B. Ho

Localization of *Helicobacter pylori* Heat Shock Protein 20

GUT 51: A-10, Supplement 11

EUROPEAN HELICOBACTER STUDY GROUP (EHSG), XV International Workshop on Gastrointestinal Pathology and *Helicobacter*, Athens, Greece. September 11 - 14, 2002.

2) R. J. Du[1], S. Y. Lui[1], B. Chaal[1], K. G. Yeoh[2], D. E. Berg[3] and B. Ho[1]

A universal epidemiological marker of *Helicobacter pylori* – Heat Shock Protein 20

Helicobacter 9(5), 2004, 507

EUROPEAN HELICOBACTER STUDY GROUP (EHSG), XVII International Workshop on Gastrointestinal Pathology and *Helicobacter*, Vienna, Austria. September 22 - 24, 2004.

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SUMMARY

Helicobacter pylori infection is associated with various gastroduodenal diseases that affect half of the world population irrespective of races and geographical regions. However, the pathogenetic mechanism of *H. pylori* infection has not been well established. Among the virulence factors of *H. pylori* reported, heat shock protein (HSP) has been identified to play an important role in protein stabilization and bacterial survival.

In this study, a 20kDa protein was identified as a homologue of HslV in the heat shock protein family and termed as heat shock protein 20 (HSP20). It has been found mainly in the spiral form of *H. pylori*. *hsp20* gene of *H. pylori* NCTC 11637 was cloned and expressed. Expressed His-tag fused recombinant HSP20 (rHSP20) in *E. coli* was purified by affinity chromatography and used as antigen to raise antibody in rabbit. HSP20 was shown to localize on the cell surface of *H. pylori* as analyzed by Western blotting and immuno-gold labeled transmission electron microscopy using rabbit anti-rHSP20 antibody.

hsp20-isogenic *H. pylori* SS1 was genetically engineered by the insertion of kanamycin cassette. Interestingly, *hsp20*-isogenic *H. pylori* retained 75% - 92% adherence ability as compared to that of the wild type bacteria by *in vitro* adhesion assay. However, when introduced separately into BALB/c mice, unlike the wild type *H. pylori*, *hsp20*-isogenic bacteria lost the ability to colonize in the stomach of the animals. This indicates that HSP20 might be involved in the colonization of *H. pylori* in mice. However, the role of HSP20 in bacterial colonization is independent of other known adhesins (e.g., OipA, HopZ and SabA) in *H. pylori*.

By co-immunoprecipitation, CagA (cytotoxin associated immuno-dominant protein) was found to interact with HSP20 in wild type *H. pylori* but not in the *hsp20*-isogenic mutant. Through RT-PCR, Western blotting and ELISA analyses, it was found that HSP20

did not affect the expression of *cagA* in *H. pylori* but influenced the presentation of CagA on the surface of *H. pylori*. These findings may imply that HSP20 could function as a "chaperon" for the presentation and stabilization of CagA in *H. pylori*, indicating the indirect association of HSP20 with pathogenesis of *H. pylori* through CagA.

The probable contribution of HSP20 in the process of *H. pylori* infection led to the DNA analysis of 227 *H. pylori* isolates which shows that *hsp20* gene is conserved in all strains tested. The phylogram based on the DNA sequences highlighted two geographical clusters: Asian and non-Asian groups. The distinctive substitution clusters of M-G-G and F-D-N clusters at 14^{th} – 16^{th} amino acid residues exhibited a strong association with these two geographical groupings as well as "close" association with PUD and NUD, respectively. The simple and unique 3 amino acid substitutions of HSP20 indicate its potential of being used as an epidemiological and gastroduodenal disease differentiating marker for *H. pylori* infection.

This study shows the novel function of HSP20 as a surface localized protein that participates in the bacterial colonization and as a chaperonic protein to the surface presentation of CagA in *H. pylori*. Furthermore, the uniqueness and simplicity of HSP20 for use in *H. pylori* epidemiology has also been demonstrated. The information obtained has thereby enriched our understanding on interactions between *H. pylori* and host.

1. INTRODUCTION

1.1 *Helicobacter pylori* and gastroduodenal diseases

Helicobacter pylori is a gram-negative, spiral-shaped microaerophilic bacteria which colonizes the human gastric mucosa. Since the successful isolation of *H. pylori* by Warren and Marshall in 1983, it has provided an opportunity for scientists to study the association of *H. pylori* with various gastro-duodenal diseases. Persistent colonization of *H. pylori* on human gastric mucosa has been strongly associated with gastric diseases ranging from gastritis, non-ulcer dyspepsia, and peptic ulcer to the increased risk of gastric cancer. As one of the human pathogens, *H. pylori* infection is the most common gastric bacterial diseases worldwide that has infected half of the world population across continents, races and age groups (Taylor & Blaser, 1991).

In the past two decades, great effort has been devoted into the study of *H. pylori* with respect to its bacteriology, physiology, genetics, pathogenesis and epidemiology of infection. Based on the studies conducted (Dunn *et al*., 1997), it is noted that *H. pylori* is a unique bacterial species that differs vastly from other bacteria. Some of these unique features are dimorphism of the bacteria cells, surface localization of cytoplasmic proteins and high genetic diversity among natural isolates (Moss & Sood, 2003).

1.2 Characteristics of *Helicobacter pylori*

Two morphological forms of *H. pylori* cells were observed: spiral form and coccoid form. Spiral-shaped *H. pylori* is the active form which is capable of colonization and infection while the coccoid form is viable but non-culturable and has been considered as the resting state of bacteria (Benaissa *et al*., 1996; Ren *et al*., 1999). Under unfavorable conditions, morphological conversion from spiral to coccoid can be observed in *in vitro*

culture such as depletion of nutrients, addition of antibiotics or stress stimuli (low pH or high temperature) (Catrenich & Makin, 1991). However, the resuscitation from coccoid to spiral has not been established in *in vitro* conditions but recovery had been reported in mice (Bode *et al*., 1993; Censini *et al*., 1996; Wang *et al*., 1997). Hence, it is a controversial issue among researchers as some regarded coccoids as dead bacterial cells (Kusters *et al*., 1997; Enroth *et al*., 1999) while others believed that coccoids are viable but non-culturable (Zheng *et al*., 1999; Ren *et al*., 1999; Saito *et al*., 2003).

The spiral form of *H. pylori* expresses a great number of proteins, which participate in various bacterial metabolic activities: e.g. cell survival & proliferation, adhesion, colonization and transportation of macromolecules. However, in the coccoid form, protein expression is significantly reduced while DNA and RNA are randomly degraded; only the basic metabolism (cell respiration, maintaining cellular integrity & DNA synthesis) is retained (Kusters *et al*., 1997; Narikawa *et al*., 1997; Costa *et al*., 1999). Therefore, it is widely believed that the dormant coccoid form is involved in the transmission of *H. pylori* infection or as *in vivo* cells responsible for treatment failure (Hua & Ho, 1996; Zheng *et al*., 1999; Andersen *et al*., 2000; Ng *et al*., 2003) while the spiral form is responsible for the pathogenesis of *H. pylori* infection (Dubois, 1995).

1.3 Virulence factors of *Helicobacter pylori*

Several proteins have been identified to be associated with *H. pylori* virulence and pathogenesis in the past decades. The most intensively studied virulence factors are cytotoxin-associated immuno-dorminant protein (CagA), vacuolating toxin A (VacA), adhesins, flagella, urease and heat shock proteins (HSPs). They act independently from

each other in the process of *H. pylori* infection but are essential for bacterial pathogenesis (Prinz *et al*., 2003).

It has been shown that CagA is one of the major virulence factors in *H. pylori* (McGee & Mobley, 1999). The gene encoding CagA is located in the "pathogenicity island (PAI)" of DNA segment that includes a cluster of 31 genes correlated with *H. pylori* specific type IV secretion system (Censini *et al*., 1996). CagA protein can be translocated into the epithelial cells to trigger a cascade of signal transduction pathways (Segal *et al*., 1999). Similarly, other major virulence factors like VacA has been demonstrated to be associated with tissue damages (Ricci *et al*., 1996). The best-known effect of VacA is its ability to induce cytoplasmic vacuoles in various eukaryotic cells (Telford *et al*., 1994).

Among the various virulence factors, outer membrane proteins (OMP) are important in mediating receptor-ligand recognition between *H. pylori* and host. Many OMPs have been identified as "adhesins" of *H. pylori* that are associated with bacterial adhesion and colonization. These include blood-group-antigen-binding adhesin (BabA) which is an adhesin of *H. pylori* interacting with the blood group antigen – Lewis antigen on gastric epithelial cells (Ilver *et al*., 1998); SabA (sialic acid-binding adhesin) that is responsible for the binding of *H. pylori* to sialyl-Lewis x antigens in gastric epithelium in humans (Mahdavi *et al*., 2002); OipA (outer inflammatory protein) and HopZ (homologue of porin) which are associated with the adhesion and colonization of *H. pylori in vitro* and *in vivo* (Yamaoka *et al*., 2002). The outer membrane associated flagella is responsible for the motility of *H. pylori* cells which is necessary for bacterial survival on the viscous mucus layer (Josenhans *et al*., 1995) while surface localized urease is an enzyme needed

to maintain a neutral pH microenvironment for the survival of *H. pylori* in the acidic stomach (Perez-Perez *et al*., 1992).

Heat shock proteins (HSPs) are another group of virulence factors, which are important for bacterial survival. HSPs are highly conserved and widely expressed in both eukaryotes and prokaryotes that are detected in folding, transporting and stabilization of proteins in cells. As one of the virulence factors, HSPs are indispensable for maintaining the normal functions of *H. pylori* proteins, assisting *H. pylori* in combating against stress and survival in the stomach (Kamiya *et al*., 1998).

1.4 Heat shock proteins (HSPs) of *Helicobacter pylori*

1.4.1 Known species of heat shock proteins in *H. pylori*

In the study of *H. pylori* heat shock proteins, several HSPs have been identified. These include 58.2 kDa - HSP60 (Dunn *et al*., 1992); 13 kDa - HSPA (Suerbaum *et al*., 1994) and 70 kDa - HSP70 (Evans, Jr. *et al*., 1992) Among these, most studies have been carried out on HSP60.

H. pylori HSP60 has been shown to be involved in protein folding as well as exporting as a chaperonin but also demonstrated immunogenic property in *H. pylori* infections. Barton *et al*. (1998) detected circulating antibodies against *H. pylori* HSP60 in patients with different gastro-duodenal diseases and the seropositivity to *H. pylori* HSP60 is strongly correlated with the degree of chronic inflammation (Vorobjova *et al*., 2001). The study of Gobert *et al*. (2004) and Yamaguchi *et al*. (1999) demonstrated that HSP60 also participates in the induction of various cytokines (interleukin-6; interleukin-8), enhances T-cell activation and interacts with Toll-like receptors (TLR-2- and TLR-4-). It

is suggested that *H. pylori* HSP60 may play a role in triggering the inflammatory process in gastric mucosa. A cross-reactive epitope was found in *H. pylori* HSP60 and its homologue in human by both Kansau & Labigne (1996) and Yamaguchi *et al*. (2000). It was speculated that *H. pylori* HSP60 might be responsible for molecular mimicry causing autoimmune response in host (Kansau & Labigne, 1996).

In addition, Amini *et al*. (1996) and Yamaguchi *et al*. (1996) reported that *H. pylori* HSP60 is translocated from cytoplasm onto the bacterial cell surface and associated with the adhesion of *H. pylori* to human epithelial cells. Surface localized HSP60 might have an essential role on the growth of *H. pylori* (Yamaguchi *et al*., 1997). It was reported to participate in the extra-cellular assembly and/or protection of other proteins against the hostile environment of stomach (Evans, Jr. *et al*., 1992; Yamaguchi *et al*., 1998).

The 13 kDa HSPA as described by Suerbaum *et al*. (1994) was shown to be related to the host immune response during *H. pylori* infection (Perez-Perez *et al*., 1996). Recent study by Eamranond *et al*. (2004) reported that the seropositivity for HSPA may be a consequence of prolonged *H. pylori* infection and is age-specific. Interestingly, the nickel binding ability of HSPA might be associated with urease (Kansau *et al*., 1996).

The other known heat shock protein is HSP70 firstly described by Evans, Jr. *et al*. (1992). The expression of HSP70 was induced in the reactive oxygen metabolite-mediated cell damage in cultured gastric mucosal cells (Hahm *et al*., 1997) but decreased upon gastric adaptation to aspirin during *H. pylori* infection (Konturek *et al*., 2001). It was also found that HSP70 might be a stress-induced surface adhesin mediating sulfatide recognition (Huesca *et al*., 1998).

Based on the knowledge acquired from heat shock proteins in *H. pylori*, it indicates that heat shock protein is an important factor that is crucial for survival of the microorganism. Furthermore, it also modulates the interactions between *H. pylori* and host such as the involvement in bacterial adherence to human epithelial cells as well as initiation of host immune response.

1.4.2 A new member of heat shock protein in *Helicobacter pylori*

Heat shock protein 20 (HSP20, HP 0515) is a newly identified member of heat shock protein family based on the open reading frame annotated by Tomb *et al*. (1997). It was predicted as a homologue of HslV in *E. coli* that is proven to be a component of ATP-dependent protease involving in the degradation of cell division inhibitor, SulA (Seong *et al*., 1999). The primary structure of HSP20 shows 49% identity to HslV while 34% similarity to human β type subunits of 20S proteosome. However, the function of this protein has not been reported.

The absence of SulA homologue in *H. pylori* and the < 50% similarity to HslV imply that HSP20 might function differently in *H. pylori*. Furthermore, the similarity between HSP20 and human β type subunits of 20S proteosome may imply a role of HSP20 in molecular mimicry of *H. pylori* infection and host immune response akin to that of HSP60. Therefore, it is useful to study the unique role of HSP20 as a protein mainly expressed in the spiral form of *H. pylori* that might be involved in the process of *H. pylori* infection or/and pathogenesis.

1.5 Objectives of this study

This project aims to characterize HSP20 and its probable function(s) in *H. pylor*. The goals of the project were:

- To clone and express *hsp20*;
- To raise specific antibody against recombinant HSP20;
- To identify the sub-cellular localization of HSP20 in *H. pylori*;
- To construct *hsp20*-isogenic *H. pylori* SS1 mutant;
- To investigate the role of HSP20 in adhesion and colonization;
- To examine proteins interacting with HSP20 in *H. pylori*;
- To explore the possibility of using HSP20 as an epidemiological marker.

2. LITERATURE REVIEW

2.1 *Helicobacter pylori* – the organism

2.1.1 Basic features of *H. pylori*

The isolation of *Helicobacter pylori* in 1983 opens a new chapter in microbiology (Marshall, 1983) Helicobacters are a new genus of bacteria, inhabiting the interface between mucosa and gastric epithelial cells. *H. pylori* is the first specie of the helicobacters genus described. It is Gram negative, microaerophilic, spiral shaped, flagellated and urease positive. It is a nutritionally fastidious microorganism forming about 1 mm transparent colony on enriched agar plate supplemented with 5 – 10% blood after 3 –5 days of incubation (Marshall and Warren, 1984). *H. pylori* is also an oxygen sensitive microorganism which only grows in the presence of 5 – 10% carbon dioxide (5-10% CO_2, 90-95% O_2) at 35 – 37°C under humidified conditions but not in regular atmosphere or under obligate anaerobic conditions (Goodwin *et al*., 1986). It is major pathogenic species in humans (Ormand *et al*., 1991).

2.1.2 Nutrition requirement of *H. pylori*

H. pylori can grow in both non-selective and selective media supplemented with antibiotics since it possesses different susceptibility to some of the antibiotics (e.g., nalidixic acid, cephalothin) (Goodwin *et al*., 1989). The addition of appropriate antibiotics developed by Skirrow and Dent (Dent and McNulty, 1988; Hazell *et al*., 1989) has improved the growth of *H. pylori* on the selective media. *H. pylori* can be cultivated on solid agar plate or in liquid broth media. The selective solid media containing antibiotics is widely used for the isolation of *H. pylori* from biopsy tissues. The growth of *H. pylori* in liquid media (generally with the supplementation of yeast extract and serum)

is relatively slower but is desirable for the studies on physiology and metabolism (Goodwin *et al*., 1986; Ho and Vijayakumari, 1993).

2.1.3 Differentiated forms of *H. pylori*

The ultrastructure of *H. pylori* is of particular interest to researchers as these features would reveal the unique structure of this pathogen and provide vital information on the correlation with pathogenesis. Based on electron microscopy study, two major morphological forms were observed: spiral and coccoid. In an early study of Benaissa *et al*. (1996), the conversion of spiral to coccoid via U-shaped transition form is clearly observed under the transmission electron microscopy. Thereafter, similar observations of morphological conversion were demonstrated in the later study by other researchers (Kusters *et al*., 1997; Costa *et al*., 1999).

The spiral shaped *H. pylori* possesses 4 – 6 polar-sheathed flagella and is highly motile (Goodwin *et al*., 1985). These basic characters (spiral shape and flagella) favor the motility of the bacteria in the viscous gastric mucus layer. The ultrathin sections of *H. pylori* under electron microscope also exhibited the typical cell wall structure of gram-negative bacterium that consists of outer and inner membrane, condensed cytoplasm containing nucleoid material and ribosome (Costa *et al*., 1999). Among the many proteins that are found to be associated with outer membrane, urease was the first identified surface located protein (Bode *et al*., 1989; Hawtin *et al*., 1990) followed by the identification of other outer membrane proteins e.g., HSP60 (Doig *et al*., 1992; Austin *et al*., 1992; Doig and Trust, 1994).

However, the round shaped coccoid form of *H. pylori* is believed to be degenerative and dead cells (Kusters *et al*., 1997). There is another group of researchers suggested that the coccoid from is viable but non-culturable (Hua and Ho, 1996; Aleljung *et al*., 1996; Saito *et al*., 2003). Substantial modifications in cell wall, surface protein profile and DNA contents were detected during the transition of coccoid (Benaissa *et al*., 1996; Costa *et al*., 1999). This phenomenon may indicate that the two forms of *H. pylori* cells may have different roles in *H. pylori* infections.

2.1.4 Morphological structure of *H. pylori*

Cell surface is an important component of extra cellular pathogenic bacteria like *H. pylori*. There are various virulence factors involving in the bacterial infection that are located or associated with the cell surface structure of *H. pylori* (Moran, 1995). Among these factors, there are two major groups that are related to adhesion and colonization as well as cell damage and bacterial survival, respectively.

A group of bacterial factors involving in the adhesion & colonization of *H. pylori* includes flagella that are responsible for motility (Jones *et al*., 1997; Clyne *et al*., 2000), urease (Tsuda *et al*., 1994; Karita *et al*., 1995), catalase & various oxidases (Harris *et al*., 2003) which are enzymes responsible for different biochemical degradation; outer membrane proteins with or without known function (Yamaoka *et al*., 2002); phospholipase (Dorrell, 1999) and adhesins (BabA) (Boren *et al*., 1993). Mutations in these genes in *H. pylori* have been reported to reduce the adherence capability or colonization ability of bacteria onto the gastric mucoca in animals.

The other major group of factors functioning in tissue damages includes vacuolating cytotoxin A (VacA); *cag* pathogenicity island (PAI) which have been shown to be related with peptic ulcer and the immune response of host (Appelmelk *et al*., 1996; Cover, 1998; Pai *et al*., 1999; Pelicic *et al*., 1999; Le'Negrate *et al*., 2001; Choi *et al*., 2001). Besides the above factors, there are some other proteins that are related to the bacterial survival, such as heat shock proteins (e.g., HSP60, HSPA, HSPR, HSP70) that are necessary for the bacteria in combating against the hostile environments (Kansau *et al*., 1996; Kawahara *et al*., 1999; Konturek *et al*., 2001; Spohn *et al*., 2002).

The findings on studying the bacterial structure revealed that *H. pylori* possesses a number of unique features necessary for colonization onto the human gastric mucus layer and survival in the hostile acid environment. Successful attachment of *H. pylori* ensures the persistence of *H. pylori* infection in gastroduodenal track. *H. pylori* infection would incite various extents of immune responses of host that is attributed to the cross-talk of factors between host and bacteria.

2.1.5 *H. pylori* and gastroduodenal diseases

H. pylori infections are closely associated with the induction of various gastroduodenal diseases such as gastritis, non-ulcer dyspepsia, gastric ulcer, duodenal ulcer and increased risk of gastric cancer. About 90% of chronic gastritis is caused by *H. pylori* (Dixon and Sobala, 1992). The association of non-ulcer dyspepsia (NUD) and *H. pylori* infection has been controversial among researchers (Pieramico *et al*., 1993). However, dyspepsia symptoms correlated with the infection of virulent *H. pylori* strains (Treiber *et al*., 2004). *H. pylori* remains the main etiological agent of peptic ulcer

including gastric ulcer (GU) and duodenal ulcer (DU) (Moss and Calam, 1993; Mauch *et al*., 1993). The eradication of *H. pylori* enhances the healing process of a bleeding peptic ulcer (Arkkila *et al*., 2003). Interestingly, the prevalence of *H. pylori* increased in patients with gastric cancer (De Koster *et al*., 1994). Thus, it signifies that the infection of *H. pylori* and related diseases is complicated, which challenges the progress of research work.

2.2 Epidemiology of *H. pylori* infection

It is reported that about 50% of the world population is infected with *H. pylori* (Taylor & Blaser, 1991). The prevalence of *H. pylori* infection varies with different regions, races, and age groups. There is a significant difference in the prevalence of *H. pylori* infection between developing and developed countries. For example, the infection rate in developing countries could be as high as 70 – 90% whereas the prevalence in the developed countries would be as low as 20 – 40% (Bardhan, 1997). In a multiethnic, Singapore, a Southeast Asian nation, it was noted that the infection rate of *H. pylori* in Chinese and Indian is higher than the other races i.e., Malay or Eurasian (Committee on Epidemic Diseases 1996; Goh, 1997; Kang *et al*., 1997), which suggests that racial differences, genetic predisposition and other environmental factors (e.g., diet) may be involved in *H. pylori* infection.

A prospective study from infancy to adulthood (Malaty *et al*., 2002) demonstrated that *H. pylori* infection could happen at the early stage of life before age 10. This indicates that the acquisition of *H. pylori* is possible during the childhood and transmitted vertically through the intrafamilial route (e.g., from parents to children). This is supported

by the findings of several studies (Taneike *et al*., 2001; Roma-Giannikou *et al*; 2003; Ng, *et al*., 2003).

2.3 Genetics of *Helicobacter pylori*

The prevalence of *H. pylori* infection varies in different graphical regions, ethnic background, socioeconomic conditions and age groups (Covacci *et al*., 1999), which could possibly be contributed by the diversified bacterial genotypes of the natural isolates. Although *H. pylori* is regarded as a big homogenous group of microorganism, the heterogeneity is widely observed among the genotype of clinical isolates and bacterial populations within the infected hosts (Blaser, 1997). The genotypic variation among *H. pylori* strains includes point mutations in conserved genes, differences in gene organization, mosaicism of conserved genes and integration of different transposable elements. The variation in bacterial population can be observed in individuals infected with more than one *H. pylori* strains ((Blaser, 1997). The formation of genotypic diversity in *H. pylori* may be related to the presence of multiple strains within one host as plural cohabitation favors the occurrence of free intraspecies recombination.

2.3.1 Genetic diversity of *H. pylori*

With the development of DNA recombination technology, the genetic diversity of *H. pylori* isolates in nature is uncovered. Upon the analyses, the comparison of DNA sequences of the same gene between *H. pylori* strains reveals that it is rare for orthologous genes from different *H. pylori* isolates to have the same sequences (panmictic structure). This finding was based on the studies of different *H. pylori*

essential genes (*ureA, ureB, flaA, flab*), virulence factor genes (*cagA, vacA*) and transposable elements (IS605) (Salaun *et al*., 1998; Suerbaum *et al*., 1998; Falush *et al*., 2001). Similar findings were also observed in the studies of other housekeeping genes of *H. pylori* such as *atpA, efp, mutY, ppa, trpC, ureI, yphC, atpD, glnA, scoB, recA* (Achtman *et al*., 1999; Maggi *et al*., 2001) or antibiotic resistance gene [*rdxA* - metronidazole resistance (Solca *et al*., 2000); *gyrA* - Ciprofloxacin resistance (Glocker & Kist, 2004)]. The detection of nucleotide diversity among different genes from different isolates indicates the existence of high level of genetic diversity in *H. pylori*.

With the availability of two complete genomes of *H. pylori*, 26695 and J99 (Tomb *et al*., 1997; Alm *et al*., 1999), it was found that the two genomes are highly similar to each other at the gene size and gene order with a limited number of discrete regions that are organized differently. When viewed in a genome wide manner, there was about 6 – 7% of the annotated genes which are strain specific but are absent from each other with no identifiable homologue in the databases (Alm *et al*., 1999).

As a major virulence factor, vacuolating cytotoxin (*vacA*) alleles show mosaic features among *H. pylori* isolates (Atherton *et al*., 1995). About 60% of *H. pylori* isolates harbor *vacA* gene, among which there are at least four different families of signal sequences (s1a, s1b, s1c and s2) and two different families of middle-region alleles (m1 and m2) (Van Doorn *et al*., 1999). Different combinations between s and m regions were identified in *H. pylori* strains isolates worldwide.

Another important virulence factor is *cag* pathogenicity island (*cag* PAI) that is a ~40 kb long chromosomal region including 31 open reading frames which encode type IV secretion system (Rohde *et al*., 2003). There is only a part of *H. pylori* isolates having

cag PAI (Mobley, 1997). The genetic diversities of both *vacA* and *cag* PAI indicate that genetic recombination and transfer may occur spontaneously among *H. pylori* strains (Atherton *et al*., 1999; Kersulyte *et al*., 1999).

The existence of different insertion sequences (IS) in genome can be the best evidence of interspecies DNA recombination and is thus valuable in bacterial taxonomy (Mahillon & Chandler, 1998). Kersulyte *et al*. (1998; 2000; 2002) and Hook-Nikanne *et al*. (1998) reported four types of insertion sequences in *H. pylori* isolates in the last few years. These insertion sequences are IS605, IS606, IS607 and IS608. The function, genetic organization and distribution of these insertion sequences have been well studied in *H. pylori* strains from different geographical regions. IS605 is the prevalent insertion sequence in *H. pylori* strains (~ 30%). The remaining types (IS606, 607 & 608) are only retained by 10 – 20% *H. pylori* isolates. Among these, IS 605 has also been reported to be involved in the deletion and insertion of *cag* PAI in bacterial genome (Bereswill, *et al*., 2000).

2.3.2 The affiliation of genetic diversity and geographical origins

Since the existence of high level of genetic polymorphism among *H. pylori* population, it is found that the clusters of *H. pylori* strains are likely to link with the status of gastroduodenal diseases, such as the presence of *cag* PAI and *vacA* alleles or other virulence factor genes that are closely associated with peptic ulcer diseases (Stephens *et al*., 1998; Arents *et al*., 2001).

Besides the association with gastroduodenal diseases, the genetic diversity of virulence factor genes, transposable elements and some of the housekeeping genes of *H.*

pylori also show distinctive affiliation with geographical origins. Falush *et al*. (2003) distinguished five major *H. pylori* populations: East Asian; Europe 1; Europe 2; African 1 and African 2, among which several subgroups were observed. Similar observations have been consecutively found in several studies carried out by different researchers around the world. Achtman *et al*. (1999) reported the existence of two weakly clonal groupings in *H. pylori* strains: Asian & Western. Ji *et al*. (2002) demonstrated a separate clustering of Chinese and Western isolates. All these findings were based on the phylogenetic analysis of a few housekeeping genes, virulence factors genes (*cagA* and *vacA* alleles). Apart from the housekeeping genes and the virulence factor genes, transposable elements (IS605, IS606 & IS608) are also a powerful marker to differentiate *H. pylori* strains from different geographical regions. Kersulyte *et al*. (2000; 2002) explored the use of IS606 and IS608 to examine the geographical distribution of *H. pylori* isolates respectively. Meanwhile, Maggi Solca *et al*. (2001) used IS605 combined with other genes found the geographical clusters of *H. pylori* strains (USA/Europe, East Asian and South African). Hence, based on these findings, it is presumed that free recombination at different gene loci has masked the evolution relationship among *H. pylori* strains.

Furthermore, Ando *et al*. (2002) found that an outer membrane protein HP0638 also exhibited geographical polymorphism and correlated with the presence of *cagA*. The geographical clusters of East Asian and Western *H. pylori* strains are able to be delineated by the in-frame & out-of-frame status that is determined by the CT dinucleotide repeat patterns of HP0638. The status is also strongly correlated with the presence of *cagA* in *H. pylori* strains. This indicates that as a surface protein, HP0638

could serve as a new genotyping system to distinguish *H. pylori* strains for epidemiological purposes.

2.3.3 Approaches for the detection of genetic diversity of *H. pylori*

Different approaches have been developed over the past decades to detect the genomic diversity of *H. pylori* isolates. The ultimate goal of these different techniques is to attempt in generating the physical maps of bacterial genome and demonstrate the extent of genetic diversity between different genomes. However, different methods have shown different sensitivity and limitation.

Before the completion of genomic DNA sequencing, several methods based on nuclease digestion were developed simultaneously for detecting the genetic diversity of bacterial genome. For example, ribotyping is a method used for analyzing the restriction enzyme digestion patterns of rRNA genes of *H. pylori* (16s & 23s rRNA) (Tee *et al*., 1992). Another enzyme-digest based method is pulsed-field gel electrophoresis (PFGE). It is used to examine the macrorestriction patterns and study the DNA homology of *H. pylori* genomic DNA (Takami *et al*., 1994; Smith *et al*., 2003).

Other than the enzyme-digest based methods, various PCR based methods were used to effectively and efficiently differentiating *H. pylori* strains. Such methods include PCR based randomly amplified polymorphic DNA (RAPD) and restriction fragment length polymorphism (RFLP). In PCR-RAPD analysis, the display of strain-specific arrays of DNA products is amplified using the arbitrarily chosen oligonucleotide primers based on genomic DNA of different *H. pylori* isolates (Marshall *et al*., 1995; Han *et al*., 2003). PCR-RFLP is to assess the restriction fragment length polymorphisms (RFLPs) in several

PCR-amplified gene segments (Akopyanz *et al*., 1992; Clayton *et al*., 1993; Fujimoto *et al*., 1994). Both methods are sensitive and useful detection approaches for analyzing a large number of *H. pylori* strains.

With the completion of *H. pylori* genome in 1997, sequencing the representative DNA segments of *H. pylori* genome is the most extensively used techniques to analyze the genetic diversity between strains accurately. This is because sequencing can provide the detailed and additional information for each DNA fragment that makes the analysis of polymorphism more rapid, direct and accurate. It is therefore the most reliable technique. A number of phylogenetic studies of *H. pylori* strains have been carried out based on different gene fragments such as housekeeping genes; virulence factor genes (*cagA, vacA*) and transposable elements (Kersulyte *et al*., 1999; Atherton *et al*., 1999; Achtman *et al*., 1999; Maggi *et al*., 2001; Falush *et al*., 2003).

The existence of high-level genetic polymorphism among *H. pylori* population and its affiliation with geographical origins or disease outcomes provide useful information for studying the evolution of *H. pylori* and epidemiology of *H. pylori* infection.

2.3.4 Evolutionary change of DNA sequences

2.3.4.1 Synonymous and non-synonymous substitutions

Synonymous substitution is the change of nucleotide that does not result in the change of amino acid encoded, whereas if the encoded amino acid is changed by the nucleotide substitution, this nucleotide substitution is termed non-synonymous. To evaluate the evolution of nucleotide sequences, it is necessary to calculate the rates of synonymous (*Ks*) and non-synonymous (*Ka*) substitutions (Nei & Kumar, 2000). The

ratio of *Ka/Ks* is an indicator to test the different selection on genes during evolution. If *Ka/Ks* = 1, gene is under the neutral selection; if *Ka/Ks* < 1 (or *Ks/Ka* > 1), gene is under negative selection and conserved; if *Ka/Ks* > 1 (or *Ks/Ka* < 1), gene is under positive selection and diverged (Hurst, 2002).

Calculation of *Ks* and *Ka* of genes has been extensively used in analyzing the phylogenetic relationship of *H. pylori* strains. In the study by Achtman *et al*. (1999) and Maggi *et al*. (2001), the *Ks, Ka* and ratio of *Ks/Ka* of some housekeeping genes from different *H. pylori* strains were employed. The *Ks* of these genes studied showed a broad range from 0% to 43% while *Ka* was in a narrow range of below 10%. The ratio of *Ks/Ka* varies among different housekeeping genes and the values were greater than 1. This implies that different housekeeping genes are under different negative selection pressures and well conserved in *H. pylori* strains.

Besides the housekeeping genes, other virulence factor genes of *H. pylori* were also studied for the rate of nucleotide substitutions. For example, *cagA* gene of different *H. pylori* strains showed high level of non-synonymous substitution rate (*Ka*) that was as high as 13% (Achtmen *et al*., 1999). Hence, the *Ks/Ka* ratio was considerably lower for this gene than that for the housekeeping genes. The other virulence factors gene is *vacA*. Atherton *et al*. (1999) examined the *Ks, Ka* and *Ka/Ks* of *vacA* alleles m1 and m2. The results showed that the greatest difference was present in the comparison between m1 and m2 but not within m1 or m2. Since the greatest *Ks* value between m1 and m2, this suggested that divergence of m1 from m2 was the most ancient.

2.3.4.2 Nucleotide divergence between populations

The estimation of DNA divergence/difference is used to evaluate the influence of DNA polymorphism between populations (Nei & Kumar, 2000). This has been extensively used in the study of *H. pylori* populations. van der Ende *et al*. (1998) utilized *cagA* gene as a marker to study the geographical groupings of *H. pylori*. Based on the calculated divergence of *cagA* at the nucleotide level (average 13.3%) and amino acids level (average 17.9%), it showed that the average differences between the Dutch and Chinese isolates was the highest. They therefore concluded that *cag*A-positive *H. pylori* populations in China and the Netherlands are distinct. Similarly, in the study of *vacA* alleles by Atherton *et al*. (1999), it was shown that the nucleotide differences between m1 and m2 clusters was 24.9% - 25.9% which was significantly higher than that within m1 or m2 cluster (both of which were below 10%). Hence, calculated nucleotide divergence is a useful parameter in analyzing different *H. pylori* populations.

2.4 Pathogenesis of *Helicobacter pylori* infection

Great diversity of *H. pylori* infections exists among different geographical regions around the world. Acquired from childhood (Malaty *et al*., 2002), *H. pylori* would be remained in the human stomach for many years as a "commensal" or an "opportunistic" pathogen. Several studies reported that only a small number of people who carries *H. pylori* would finally develop clinical symptoms. It is believed that the pivotal feature of *H. pylori* might be its capability to persist within the host rather than to damage the host tissue (Mobley, 1997; Marshall, 2002; Lamarque & Peek, 2003; Blaser & Atherton, 2004).

In the past ten years, much effort has been devoted into the study of *H. pylori* pathogenesis. The identification of potential virulence factors is intriguing. The identified virulence factors can be sorted into different groups mediating different biochemical reactions. This includes the outer membrane proteins: adhesins (e.g., BabA) (Boren *et al*., 1993), immunogenic antigens (CagA, urease, LPS) (Tummuru *et al*., 1993; Tsuda *et al*., 1994; Karita *et al*., 1995; Appelmelk *et al*., 1996) proteins involved in adherence and colonization (HP0638; phospholipase A) (Dorrell *et al*., 1999; Yamaoka *et al*., 2002); cytotoxins (CagA, VacA, NAP) (Evans, Jr. *et al*., 1995; Pelicic *et al*., 1999; Choi *et al*., 2001; Le'Negrate *et al*., 2001); motility machinery (flagella) (Jones *et al*., 1997; Clyne *et al*., 2000); anti-oxidant related proteins (catalase, SOD) (Seyler, Jr. *et al*., 2001; Harris *et al*., 2003) and others cytoplasmic proteins, e.g., heat shock proteins (Kansau *et al*., 1996; Kawahara *et al*., 1999).

2.4.1 Adherence and colonization of *H. pylori*

Prior to the expression of disease by *H. pylori*, adhesion and colonization of the bacteria on the gastric mucus layer is essential. Hence, bacterial adherence and colonization become the prerequisite for the persistence of *H. pylori* on the gastric epithelial cells and the subsequent immune responses of the host. To begin with, adherence of *H. pylori* on the gastric epithelium is the initial step before colonization. In fact, *H. pylori* possesses different apparatus to cope with the intricate structure of the gastric mucus. It has been reported that the flagella help to "swim' in the viscous gastric environment (Luke *et al*., 1990). Urease hydrolyzes urea to create a neutralized microenvironment in the acid-pH conditions (Labigne *et al*., 1991). Adhesins mediate receptor-ligand recognition to

facilitate the attachment of bacteria onto the gastric epithelium (Tomb *et al*., 1997). Beside the adhesins, there are other factors e.g., phospholipase A, SOD, catalase to join the stabilization of bacteria on gastric epithelia (Langton & Cesareo, 1992; Mori *et al*., 1997; Figura & Valassina, 1999).

2.4.1.1 Role of flagella

H. pylori is endowed with 4 – 6 polar based sheathed flagella. Luke *et al*. (1990) showed that flagellum is a complex of outer membrane polypeptides of various molecular weights (MW) under electron microscopy and SDS-PAGE. Two major flagellin species, FlaA and FlaB with MW of 56 kDa and 57 kDa respectively were found in the flagellar filaments (Kostrzynska *et al*., 1991). It was shown that *flaA* and/or *flaB* –disrupted *H. pylori* mutants demonstrated the loss of motility function and failure of such bacterial mutant to colonize in animal stomach (Josenhans *et al*., 1995; Andrutis *et al*., 1997). Another study conducted on examining the antibody against *H. pylori* showed that there was a cross-reaction of monoclonal antibody against flagella and human tissue. This implies the possible involvement of flagella proteins in autoimmune responses of host during *H. pylori* infections (Ko *et al*., 1997).

2.4.1.2 Role of Urease

Urease is one of the most extensively studied proteins in *H. pylori*. Two urease subunits genes were initially cloned in 1990 by Clayton *et al*. (1990) that encode 26 kDa (subunits A) and 60 kDa (subunit B), respectively. The urease gene cluster of *H. pylori* was revealed by the complete sequenced bacterial genome in 1997 (Tomb *et al*., 1997).

Study by Voland *et al*. (2003) summarizes the functional elements of urease gene cluster: encoding catalytic subunits (*ureA/B*), an acid-gated urea channel (*ureI*) and accessory assembly proteins (*ureE-H*).

The constructed *H. pylori* isogenic urease mutant demonstrated that urease may protect *H. pylori* against acidic environment of stomach (Segal *et al*., 1992). Later studies found that urease would affect the colonization of *H. pylori* in nude mice (Tsuda *et al*., 1994; Karita *et al*., 1995) but did not act as an adhesin (Clyne & Drumm, 1996).

The antigenicity of urease in bacterial infection and host immune response has been well documented. It was found that urease protein is an immunodorminant antigen in *H. pylori* (Stacey *et al*., 1990). The antigenic epitope of urease was identified by Hirota *et al*. in 2001, which showed its capability of enticing the production of neutralizing antibody in host.

2.4.1.3 Role of adhesins

Adhesins play a vital role in *H. pylori* infection and the development of gastroduodenal diseases as they are integrated components of bacterial adherence. Many adhesins have been identified or predicted by the annotation of ORF (open reading frame) in *H. pylori* genome (Tomb *et al*., 1997). Adhesins are mainly outer membrane proteins that mediate receptor-ligand interaction between bacteria and host escorting the adhesion of *H. pylori* on the mucus layer. Among which, blood group A antigen-binding adhesin (BabA) is extensively studied. BabA is involved in the binding to the blood group antigen Lewis b surface epitopes of host and encoded by allelic *babA2* gene (Boren *et al*., 1993; Ilver *et al*., 1998).

Another newly identified adhesin is SabA (sialic acid-binding adhesin) for binding to sialyl-Lewis x antigens in gastric epithelium in humans (Mahdavi *et al*., 2002). The adherence of *H. pylori* to sialylated glycoconjugates expressed during chronic inflammation thereby contributing to the virulence and the extraordinary chronicity of *H. pylori* infection (Mahdavi *et al*., 2002).

Besides the two adhesins described above, the other adhesins reported to be involved in the adherence of *H. pylori* onto the gastric epithelium are OipA (outer inflammatory protein, HP0638) (Yamaoka *et al*., 2000); HopZ (homologue of porin) (Peck *et al*., 1999). It is noted that there are different number of CT dinucleotide repeats in the signal sequences of these adhesins genes (*oipA*, *hopZ* and *sabA*) which determine the functional status of genes. It was reported that when the CT dinucleotide repeats are in frame, the function of expressing the adhesins is turned "on". However, upon insertion or deletion would have caused these repeats out of frame, the function is then turned "off" (Yamaoka *et al*., 2000). This functional status ("on" / "off") of *oipA*, *hopZ* and *sabA* were found to affect the adherence and colonization of *H. pylori* (Yamaoka *et al*., 2000).

2.4.1.4 Other factors related to the colonization of *H. pylori*

The additional crucial steps after adhering to the gastric epithelium for bacterial establishment would be colonization so as to facilitate the proliferation of *H. pylori* in the host. Other than the adhesive proteins identified, some other proteins have also shown their relevance to colonization of *H. pylori* in stomach, like pospholipase A (Dorrell *et al*., 1999), SOD, catalase (Seyler, Jr. *et al*., 2001), γ-glutamyl transpeptidase (GGT) (Chevalier *et al*., 1999).

Phospholipase A (PldA) of *H. pylori* is an enzyme capable of hydrolysing gastric mucosal phospholipids that is believed to be involved in the mucus damage and ulceration (Langton & Cesareo, 1992). The mutagenesis study of *H. pylori* PldA (phospholipases A) demonstrated that phospholipase plays a role in bacterial colonization in terms of damaging the gastric mucus and tissue but not the host immune responses in mice (Dorrell *et al*., 1999).

Superoxide dismutase (SOD), a major defense mechanism against oxidative damage, catalyzes the breakdown of superoxide radicals while catalase catalyzes the reaction to decompose the hydrogen peroxide. Both enzymes are strictly cytoplasmic localized proteins in other bacteria. However, it was found that these two enzymes are secreted onto the cell surface of *H. pylori* (Mori *et al*., 1997). The isogenic *H. pylori* mutants of these two proteins and the absence of the capability of colonization in mice suggest that the ability of the anti-oxidative agent might affect the bacterial colonization and bacterial survival (Seyler, Jr. *et al*., 2001; Harris *et al*., 2003).

The study of Chevalier *et al*. (1999) showed that γ-glutamyl transpeptidase (GGT) is essential for bacterial colonization in mice. However, its role is controversial as shown in a later study carried out by McGovern *et al*. (2001) where GGT was reported to affect the *H. pylori* load in colonized piglet and mice but not colonization. Other enzymes (e.g., alcohol dehydrogenase, neuraminidase) could promote tissue erosion and ulceration by destroying the integrity of mucus, inducing lipid peroxidation and the like, which might be also closely related to bacterial adhesion and colonization (Figura, 1997).

Host factor like secretory IgA has been reported to inhibit the adhesion of *H. pylori* (Falk *et al*., 1993). Similarly, host super oxide dismutase (SOD) had shown to be

depleted significantly in the ulcer edge of peptic ulcer patients (Klinowski *et al*., 1996). In the process of *H. pylori* adhesion and colonization, chemotaxins (e.g. IL-8) are also involved in host tissue damage (Mobley 1997). In addition, *H. pylori* promote PMN adhesion to endothelial cells in gastrointestinal track (Yoshida *et al*., 1993) while gastric epithelial cells acted as antigen presenting cells in activating proinflammatory cytokines (Maekawa *et al*., 1997). These two factors may play important roles in the humoral immune response of host during *H. pylori* infection.

Based on the knowledge obtained, it is noted that a number of factors are involved in the adhesion and colonization of *H. pylori*. It further addresses that the successful establishment of *H. pylori* on gastric mucus is the prerequisite for the persistence of *H. pylori* infection.

2.4.2 Major virulence factors of *H. pylori*

2.4.2.1 *cag* Pathogenicity Island (cag PAI)

It is a pathogenicity island (PAI) of approximately 40 kb encoding 31 genes and present in a subset of *H. pylori* (Mobley, 1997). *cag* PAI is associated with the virulence of *H. pylori* since it encodes a putative secretory system – type IV secretion system that is responsible for translocating proteins (e.g., CagA) from *H. pylori* into the host cell (Censini *et al*., 1996). The strains containing the PAI are more virulent than those without and it has shown a positive association with peptic ulcer and gastric cancer (Covacci *et al*., 1993; Blaser *et al*., 1995; Akopyants *et al*., 1998; Figura & Valassina, 1999). *cag* PAI is likely to have acquired horizontally and integrated into the chromosomal of *H. pylori* that is involved in the activation of cell signal transduction cascade of host including the induction of the transcription factor NF-κB with the consequent secretion of

proinflammatory cytokine IL-8; in addition, to inducing the rearrangement of cytoskeleton with pedestal formation (Segal *et al*., 1996, 1997).

cagA gene product was the first identified protein of *cag* PAI which encodes a cytotoxicity associated immunodorminant antigen with MW of about 128 kDa (Tummuru *et al*., 1993). As a cytotoxin associated protein and major virulence factor, the remarkable feature of CagA is its immunogenicity that elicits strong immune response in the host during *H. pylori* infection. High titer antibody against CagA could be detected in the patients with various gastroduodenal diseases, especially those with peptic ulcer disease (DU & GU) and gastric cancer patients (Klaamas *et al*., 1996; Ching *et al*., 1996; Matsukura *et al*., 1997; Miehlke *et al*., 1998; Vaucher *et al*., 2000). It suggests the importance of CagA in the pathogenesis of *H. pylori* infection.

On the other hand, CagA has been reported to disrupt the tight junction proteins of host cells, which occurs at the site of *H. pylori* attachment (Amieva *et al*., 2003). Hence, as a cytotoxin associated antigen, the vital role of CagA in *H. pylori* is highly significant in the pathogenesis of *H. pylori* infection.

2.4.2.2 Vacuolating cytotoxin (VacA)

Vacuolating cytotoxin is another extensively studied virulence factor in *H. pylori* which induces cytoplasmic vacuolation in eukaryotic cells. The mature cytotoxin is 88kDa which is further cleaved into two subunits: p33 and p55. The subunit p55 is important in binding of VacA to host cells (Reyrat *et al*., 1999). Two receptor tyrosine phosphatases RPTP alpha and RPTP beta identified as the receptors of VacA in gastric cell lines (Padilla *et al*., 2000; Yahiro *et al*., 2003). VacA also can be translocated

through the "IgA protease-type of exoprotein similar system" from bacterial membrane into the infected host cell cytosol. This causes a lesion of the late endosomal/lysosomal compartments by altering the protein trafficking (Schmitt & Haas, 1994; Fischer *et al*., 2001). However, *vacA* isogenic *H. pylori* mutant did not show deficiency of bacterial colonization in animals (Eaton *et al*., 1997; Wirth *et al*., 1998). Interestingly, the antibody level against VacA was shown to be higher in patients with peptic ulcer (Donati *et al*., 1997). The latest study shows that VacA will inhibit the activation of T lymphocytes by mimicking the activity of the immunosuppressive drug FK506 thereby inducing the local immune suppression during the chronic inflammation of *H. pylori* infection (Gebert *et al*., 2003). These results showed that VacA is one of the major virulence factors related to the tissue damage during *H. pylori* infection.

2.4.2.3 Other virulence factors

Other factors involved in the pathophysiological processes of *H. pylori* infection are neutrophil-activating protein (NAP) that plays an important role in inducing gastric inflammatory response of *H. pylori* (Evans, Jr. *et al*., 1995) and alkyl hydroperoxide reductase (AhpC), a protein catalyzing the reduction of organic peroxides, functions to assist the microaerophilic pathogen to survive oxidative and aerobic stress (Baillon *et al*., 1999). Besides these, other probable virulence factors like mucinase (homologue of Hap of *V. cholerae*), Lewis antigens and LPS have been reported (Moran, 1996; Figura, 1997).

Based on the findings obtained, it is apparent that the progression of *H. pylori* infection is a complicated but elaborately regulated process where multiple factors are

involved. Although great effort has been incessantly dedicated in the study *H. pylori*, the pathogenic mechanism of *H. pylori* infection remains a mystery.

2.5 Surface localized proteins of *Helicobacter pylori*

As an extracellular pathogen, the communication between *H. pylori* and host cells is vital. During the process of *H. pylori* infection, the interactions between bacteria and host are throughout the entire progress and directly related to the severity of inflammation induced. In this process, surface structures of *H. pylori* and host are the core components. A great variety of bio-molecules are present on the cell surface of *H. pylori*. These include small phospholipid components forming the double layer membrane scaffold; proteins with various sizes inserted into the double layer or protruded facing internally or externally and carbohydrates coat for protection. Generally, protein molecules faced internally would be coupled with other molecules to trigger the signal cascade while the proteins faced externally would receive the stimuli and pass down the information. Therefore, the surface proteins of pathogens are crucial messengers in transferring the information between microorganism and host during the infection.

In essence, surface proteins of *H. pylori* are important for the implementation of pathogenesis. The surface proteins of *H. pylori* are unique among bacterial species, where the surface localized cytoplasmic proteins is an unusual phenomenon restricted within *H. pylori* strains. There are a number of proteins which are strictly distributed in cytoplamsic regions in other bacterial species but found localized on the surface in *H. pylori* bacterium cells (Hawtin *et al*., 1990; Evans, Jr. *et al*., 1992; Mori *et al*., 1997). It is widely believed that this is functionally significant and important for *H. pylori*.

Urease is one such protein that was first found to be surface associated in *H. pylori* cells. The surface localized urease was identified by Hawtin *et al*. (1990) using indirect immunogold electron microscopy labeling technique. Due to the urease enzyme activity, it was demonstrated that generation of ammonia from urea hydrolyzed by urease in the stomach would cause damages to the gastric mucosa (Murakami *et al*., 1990). Further study has shown that surface localized urease possesses capability of activating monocytes for stimulation of IL-8 production in epithelial cell lines (Harris *et al*., 1996).

HSP60 homologue was later found to be associated with urease enzyme in the crude preparation of bacterial surface fraction (Evans, Jr. *et al*., 1992). Urease-associated HSP60 was believed to participate in the extracellular assembly and/or protection of urease against inactivation in the hostile environment of the stomach. Surface localized HSP60 also showed its ability to entice the antibody and cytokines responses (Sharma *et al*., 1997). In addition, further study showed that cell surface HSP60 would mediate sulfatide recognition by *H. pylori* under the stress conditions (Huesca *et al*., 1996).

Many cell surface associated proteins, e.g., catalase and SOD (Mori *et al*., 1997) are important for the bacterial survival under the stressed conditions (acidic and/or oxidative). The surface localized proteins are not only related to the bacterial survival and resistant against the hostile environment, but also closely corelated with the host immune responses (antibody and/or cytokines productions).

The importance of surface localized proteins signifies a particular "hiding" mechanism for the translocation of proteins from cytoplasm to cell surface in *H. pylori*. Phadnis *et al*. (1996) found that urease and HSP60 became outer-membrane-associated after the autolysis of bacteria cells as revealed by cryo-immunoelectron microscopy.

They also found that *H. pylori* underwent spontaneous autolysis during *in vitro* cultivation suggesting that the surface structure of *H. pylori* must be unique for the absorption of cytoplasmic proteins. The bacterial autolysis and surface associated urease & HSP60 were also demonstrated in human gastric biopsies (Dunn *et al*., 1997) thereby further strengthening the possible programmed bacterial autolysis process *in vivo*.

The proposed altruistic autolysis model of *H. pylori* was prevailing for a period of time until 1998 when Vanet and Labigne (1998) showed the evidence for the existence of specific secretion mechanism. In their study, urease and HSP60 homologue were utilized for the analysis. Relying on the subcellular fractionation approach associated with quantitative Western blot analyses, Vanet and Labigne showed that the releasing process of cytoplamic proteins is selective. Some cytoplasmic proteins (beta-galactosidase homolog) were strictly present in the cytoplasmic fraction but not urease & HSP60, which were present in both cytoplasmic and surface factions. Hence, they suggested that a specific selective mechanism(s) is involved in the secretion of some *H. pylori* antigens while bacterial autolysis does not seem to make a major contribution.

However, many researchers observed that both mechanisms would exist for this particular process in *H. pylori* cells: non-selective (autolysis, spontaneously occurred during bacterial cultivation *in vitro*) and selective (by specific secretory system). Whichever mechanism is involved in the surface associated cytoplasmic proteins, releasing proteins extracellularly by *H. pylori* would be an important adaptation that facilitates the persistence of *H. pylori* in the human gastric mucus layer. The entry of these proinflammatory proteins from *H. pylori* into the gastric mucosa may contribute to the induction of a mucosal inflammatory response (Cao *et al*., 1998; Schraw *et al*., 1999).

2.6 Immuno-labeled transmission electron microscopy (TEM) and protein localization in *Helicobacter pylori*

With the development of antibody techniques and electron microscope, immuno-labeled electron microscopy becomes a useful tool to display the ultrastructure location of protein molecules in cells or tissues. It is widely used in studying the localization of proteins in eukaryotes and prokaryotes (Herrera, 1992). Immunolabeling TEM is the most commonly used technique to visualize the precise localization of molecules in cells. Protein A-gold conjugates are generally used as a probe for immuno-staining where the gold particles can be easily seen under electron microscopy (EM).

Immuno-gold labeled TEM was widely used in studying the proteins localization in *H. pylori.* In 1990, Hawtin *et al*. used the monoclonal antibodies of urease to image the localization of urease in *H. pylori*. Later, Drouet *et al*. (1991) and Drouet *et al*. (1993) demonstrated the surface localization of 19 kDa outer membrane protein and polysaccharide of *H. pylori*. The localization of various proteins of *H. pylori* was resolved by different researchers using immuno-gold labeling TEM techniques (Lelwala-Guruge *et al*., 1993; O'Toole *et al*., 1994; Jones *et al*., 1997; Chirica *et al*., 2002). Phadnis *et al.* (1996) improved on the immuno-gold electron microscopy by using cryo-immuno-electron microscopy show the surface association of cytoplasmic proteins in *H. pylori* where urease and HSP60 of *H. pylori* were found to localize on the bacterial cell surface and cytoplamsic regions.

It can be shown that the immuno-labeling TEM is a powerful tool in studying the localization of proteins in *H. pylori*. With this technique, it can clearly visualize the exact

location of macromolecules in *H. pylori*. The localization of molecules provides important information on their possible functions in *H. pylori* infection.

2.7 Homology modeling of protein structure

Homology modeling is also called comparative protein modeling or knowledge-based modeling. It is the process where a 3D model of a target protein sequence is built based on a homologue (preferably with at lest 30% similarity) with experimentally resolved protein structure (by X-ray crystallography or nuclear magnetic resonance, NMR) (Baker & Sali, 2001). With the development of computer (and or information) technology, availability of protein database (PDB) and experimentally resolved protein structures, it is possible for researchers to carry out the prediction of protein structure based on the similarity between homologues to facilitate the study of function of proteins.

In recent years, homology modeling of protein structure in *H. pylori* has been carried out. In the study carried out by Marsich *et al*. (2002), a gene encoding an autolytic enzyme was cloned and its structure was predicted by molecular modeling. This gene was almost identical to the HP0339 of *H. pylori* 26695. It is a homologue of bacterial bacteriophage T4 lysozyme showing >95% identity to each other. The protein structure encoded by this gene was predicted using molecular modeling based on the resolved structure of bacteriophage T4 lysozyme. Through the modeling, this novel protein of *H. pylori* showed typical lysozyme folding and catalytic cleft suggesting that this protein belongs to the bacterial lysozyme family.

Similarly, DnaA of *H. pylori* has been identified to be involved in the initiation of *H. pylori* chromosome replication. Although DnaA of *H. pylori* is a homologue of DnaA in

E. coli, it showed lower DNA binding specificity. Through the comparative molecular modeling of DnaA (36% identify to DnaA of *Aquifex aeolicus*), it showed that there are 9 residues within the binding domain of this protein that are possible determinants for the reduced *H. pylori* DnaA binding specificity (Zawilak *et al*., 2003).

2.8 Gene mutagenesis study in *Helicobacter pylori*

The advance development of molecular biological techniques in early 1990's has made it possible to study and understand the function of protein in *H. pylori* with the construction of specific isogenic gene mutant. The isogenic urease-negaitve *H. pylori* mutants were initially constructed by Ferrero *et al*. (1992) through allelic replacement. In the targeting vector, cloned urease genes (*ureA* & *ureB*) were disrupted by the insertion of a mini-Tn3-Km transposon where the kanamycin resistant gene (Km) was derived from *Campylobacter*. The allelic exchange occurred between targeting vector and chromosome of *H. pylori* at urease gene loci after electrotransformation. The mutant was resistance to kanamycin and identified using Southern hybridization and immunoblot. It was noted from this study that only part of *H. pylori* isolates were competent for transformation. This genetic engineered urease mutant was helpful in the study on the role of urease in the pathegnesis of *H. pylori* infection.

It was reported that aflagellated mutants of *H. pylori* were generated using transposon shuttle mutagenesis (Haas *et al*., 1993). In their study, it was found that some of *H. pylori* clinical isolates were naturally competent for genetic transformation with the transformation frequency of 5×10^{-4} and 4×10^{-6}. The targeting vector contained the cloned *flaA* gene that was interrupted by insertion of TnMax1, a mini-Tn1721 transposon

carrying a modified chloramphenicol-acetyltransferase gene (catGC cassette). Similarly, the mutant was obtained after natural transformation and identified using Southern blotting and immunoblotting. The phenotypical characterization was further demonstrated under the electron microscopy. From this study, it provides the opportunity to construct transposon shuttle mutagenesis for *H. pylori* based on natural competence.

Over the past decade, various isogenic genes mutants of *H. pylori* through allelic exchange mediated by electroporation or natural transformation have been constructed. These included *nixA* isogenic mutant which has reduced nickel transport and urease activity (Bauerfeind *et al*., 1996); ABC transporter gene mutant which affects production of a catalytically active urease (Hendricks & Mobley, 1997); aliphatic amidase gene mutant which reveals the role of amidase in intracellular ammonia production of *H. pylori* (Skouloubris *et al*., 1997); *cdrA* gene mutant was shown to be related to the cell division of *H. pylori* (Takeuchi *et al*., 1998); *rpoN* (encoding RNA-polymerase sigma54 subunit in *H. pylori*) gene mutant (Fujinaga *et al*., 2001) and catalase gene mutant (Harris *et al*., 2002). From these studies, it is noted that allelic exchange is a primary yet only method in constructing the isogenic mutant of *H. pylori*. The selection markers commonly used are kanamycin resistant and chloramphenicol-acetyltransferase (catGC cassette) genes.

2.9 Animal model of *Helicobacter pylori*

As a human pathogen, the study of *H. pylori* in animals is helpful in the understanding on/of the pathogenesis of *H. pylori* infection *in vivo*. Therefore, the establishment of animal models for *H. pylori* infection is important. Pig model was

initially established to study the gastritis type B induced by *H. pylori* by Engstrand *et al*. (1990). In this study, the pigs were intragastrically inoculated with suspensions of 10^7 to 10^{10} CFU of *H. pylori*. The infected pigs were examined for *H. pylori* infection up to 12 weeks. *H. pylori* and antibody against *H. pylori* was detected in 11 out of 15 pigs infected throughout the observation time. Furthermore, the superficial, focal gastritis was developed in the infected pigs observed immunohistologically. This indicates the usefulness of animal model in studying *H. pylori* related human diseases.

Meanwhile, the ferrets infected with *H. mustelae* became an animal model for *H. pylori* induced gastritis in humans (Fox *et al*., 1990). The colonization of *H. mustelae* on gastric mucosa in ferrets was 100% and heavily at the duodenum and antrum. Significant immune response to this organism was detected. Superficial gastritis was noted. However, ferrets lack the polymorphonuclear-cell response that is generally seen in the active chronic gastritis typically described with *H. pylori* gastritis in humans. The lesion in ferrets does closely resemble the diffuse antral gastritis seen in human with *H. pylori* induced gastritis. The ferret model provides the possibility to study multiple host and environmental variables during *H. pylori* colonization and the progression of gastroduodenal diseases.

Mice infected with *H. felis*, a microorganism isolated from cat and found to be closely related to *H. pylori* became a small animal model for *H. pylori* (Lee *et al*., 1990). Similarly, significant histopathology was seen in *H. felis* infected mice. During the period of infection, infected mice showed progression from acute inflammation to persistent acute on chronic inflammation (active chronic) that is similar as seen in human infection with *H. pylori*. Since then, *H. mustelae* infected gnotobiotic rats (Fox *et al*., 1991); *H.*

pylori infected piglets (Bertram *et al*., 1991; Eaton *et al*., 2001); *H. pylori* infected monkeys (Fujioka *et al*., 1994); *H. pylori* infected rats (Ross *et al*., 1992) and guinea pigs (Shomer *et al*., 1998) became animal models for studying *H. pylori* infection and the pathogenesis of human gastroduodenal diseases.

In order to develop a convenient and experimental rodent model, both BALB/c nude and BALB/c euthymic mice were used. Karita *et al*. (1991) challenged them with 2×10^8 *H. pylori*. After 20 weeks incubation, the animals were infected with *H. pylori*. Resulting from colonization of *H. pylori*, gastritis and duodenitis were observed. Beside the BALB/c mice used, C57 mice infected with *H. pylori* had also been used as an animal model for studying *H. pylori* infection (Smythies *et al*., 2000; Suresh *et al*., 2000). However, C57 mouse model showed different susceptibility to *H. pylori* infection as compared with BALB/c (Smythies *et al*., 2000; Suresh *et al*., 2000). As a small animal model, the murine model for *H. pylori* infection was not only used for studying *H. pylori* associated diseases but also used to study the immune responses or cytokines production of host during *H. pylori* infection (Wadstrom *et al*., 1994; Ferrero *et al*., 1995; 1998). Compared with other animal models, small rodent like mouse provide an efficient way to study the pathogenesis of *H. pylori*. The infection of *H. pylori* in mice is rapid (could occur as short as 2 weeks after inoculation), stable and reproducible. This animal model is not only "time-saving" but also "cost-saving" for studying the *H. pylori* and related human diseases. Since the colonization of *H. pylori* in mouse could occur as early as 2 weeks of post-inoculation, it is especially useful to differentiate the factors that affect the early events of *H. pylori* infection *in vivo*. However, there are some limitations in the use mouse model. Karita *et al*, (1994) noted that only temporary colonization was detected in

the "non-germ free" mice as compared to the "germ free" mice. In addition, lower bacterial count was found in the euthymic mice than that of the athymic mice (Karita *et al*, 1994). Variations in the severity of disease induced by *H. pylori* infection were observed when different types of mouse strains used (Lee, 1995; Mohammadi *et al*., 1996).

3. MATERIALS & METHODS

3.1 Propagation of bacteria and cell lines

3.1.1 *H. pylori* and *E. coli*

H. pylori strains used in this study included: *H. pylori* NCTC 11637, *H. pylori* SS1 (mouse adapted strain) and 225 clinical *H. pylori* isolates obtained from different geographical regions. Among these, 103 strains were isolated from Singapore and 122 strains were from 9 different countries (Japan, 43; India, 6; Hong Kong, 6; Sweden, 16; Spain, 14; Peru, 12; Lithuania, 12; Costa Rica, 9 and Australia, 4).

H. pylori can grow either in broth medium or on agar plate. In liquid culture, *H. pylori* was grown in liquid culture of brain heart infusion (BHI, Oxoid) broth supplemented with 0.4% yeast extract (Oxoid) and 10% horse serum (Gibco). On solid medium, *H. pylori* was cultured on chocolate blood agar (CBA) plates containing Blood Agar base No. 2 (Oxoid) supplemented with 5% horse blood (Quad Five). The CBA plates were supplemented with four antibiotics: vancomycin 3 μg/ml, trimethoprim 5 μg/ml, nalidixic acid 10 μg/ml and amphotericin 2μg/ml (All antibiotics were purchased from Sigma). The broth and solid cultures were incubated at 37°C in an atmosphere of 5% CO_2 (95% O_2) in a humidified incubator (Forma Scientific) for 3 days. All culturing procedures were carried out under biosafety level 2 conditions within a biohazard cabinet.

E. coli Top10 and BL-21 strains were used in this study. *E. coli* was grown in LB broth or on agar plates (Appendix 9). The growth of *E. coli* cells transformed with plasmid DNA was cultured in LB medium supplemented with the appropriate concentration of corresponding antibiotics (i.e. ampicilin 50 μg/ml or kanamycin 25 μg/ml).

3.1.2 Gastric carcinoma cell lines

Two gastric carcinoma cell lines used in this study were KATO III and AGS. Both were obtained from ATCC (USA). All culturing procedures were carried out under biosafety level 2 conditions in tissue culture room within a biohazard cabinet.

KATO III cell line (semi–adherent cell line) was grown in RPMI 1640 medium (Sigma) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin-streptomycin (Sigma). The cells were incubated at 37°C with 5% CO_2 for 3 – 4 days. Sub-culturing was carried out when confluent monolayer of cells was formed. The grown cells were digested with 1 × trypsin solution (5mg/ml) (Sigma) for 1 minute and resuspended in appropriate volume of fresh culture medium. The ratio of sub-culturing was 1:2 – 1:3.

AGS cell line (adherent cell line) was grown in Ham's F12K medium (Gibco) [2 mM L-glutamine (Sigma) adjusted to contain 1.5 g/L sodium bicarbonate] supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Similarly, the cells were kept at 37°C with 5% CO_2 incubator for 2 – 3 days. When monolayer of cells formed, the grown cells were digested with 1× trypsin solution (5mg/ml) for 5 minutes and resuspended with fresh Ham's F12K medium. The ratio of sub-culture was carried out at 1:3 – 1:5.

The cells grew either in disposable flasks (25 cm^2 or 75 cm^2) or 6-well plates (NUNC).

3.2 Genomic study

3.2.1 Extraction of *H. pylori* genomic DNA

Genomic DNA of *H. pylori* isolate was extracted according to the method described by Hua *et al*. (1999)**.** Briefly, the 3-day old *H. pylori* plate culture was harvested and transferred into a microfuge tube and washed twice with TE buffer (pH 8.0, Appendix 16) at 8000 × g for 5 minutes. The cell pellet (about 10^9 cells) was resuspended in 300 - 400 µl TE buffer. The cell suspension was treated with 100 µl of 10 mg/ml lysozyme (Sigma) at 37°C for 30 minutes to break down the cell wall and outer-membrane. The resultant spheroplasts were then lysed with 100 µl of 10% SDS for additional 30 minutes at 37°C followed by further treatment with 5 µl of 10 mg/ml proteinase K (Gibco) at 56°C for 1 hour. The DNA solution was extracted twice with equal volume of phenol and once with equal volume of chloroform. DNA was precipitated overnight with two volumes of absolute ethanol and 1/10 volume of 3 M sodium acetate (pH 4.8) at - 20°C. The DNA was pelleted at 12000 × g for 15 minutes and washed twice with 70% alcohol. The dried DNA pellet was resuspended in appropriate volume of TE buffer (pH 8.0) and digested with RNase (20 µg/ml) at 37°C for 30 minutes. The DNA concentration was measured spectrophotometrically at 260 and 280 nm.

3.2.2 Transformation of *E. coli* cells

3.2.2.1 Preparation of *E. coli* competent cells

Calcium chloride was used in the procedure of preparation of competent cells as described by Sambrook *et al*. (1989). A single bacterial colony of *E. coli* was inoculated into 50 ml of LB medium in a 500 ml (PYREX) flask and incubated at 37°C for

approximately 3 hours on a shaker incubator (B. Braun) at 200rpm. The growth of bacteria was monitored by measuring at OD_{600} every 30 minutes. The cells were harvested when OD_{600} reached ~ 0.3.

Bacterial culture was then transferred into a sterile disposable ice-cold 50 ml centrifuge tube (Falcon) and cooled on ice for 10 minutes. Cell pellet was collected at 5000 × g for 5 minutes at 4°C. The resultant supernatant was discarded. Excess liquid was drained by inverting the tube on a pad of absorbent paper. Cell pellet was resuspended by swirling in 30 ml of ice-cold 0.1M $CaCl_2$ (filtered with 0.2 μm sterile filter) and incubated on ice for 30 minutes. The $CaCl_2$-treated cells were spun down at 5000 × g for 5 minutes at 4°C. The cell pellet was resuspended in 2 ml ice-cold 0.1 M $CaCl_2$ and the cell suspension was aliquoted in 200 μl amount. The competent cell aliquots were stored at – 80°C until use.

3.2.2.2 Transformation of plasmid DNA with insert

DNA (no more than 50 ng in a volume of 10 μl or less) was added into 200 μl competent cell suspension and mixed by gentle tapping. The mixture was placed on ice bath for 30 minutes. The competent cells were then heat shocked by incubating at 42°C for exactly 90 seconds and rapidly transferred the tube onto ice bath for 1 – 2 minutes. Aliquot of 800 μl LB broth was added into the DNA-cell suspension and incubated at 37°C for 30 – 45 minutes with shaking (200 rpm). From the DNA-cell medium suspension, 200 μl was withdrawn and spread onto a fresh LB agar plate containing appropriate concentration of antibiotics (i.e. ampicilin 50 μg/ml or kanamycin 25 μg/ml) and incubated overnight at 37°C.

3.2.3 Mini-preparation of plasmid DNA

The protocol for plasmid DNA preparation was carried out as described by Sambrook *et al*. (1989). A single colony of plasmid transformed *E. coli* was inoculated into 5 ml of LB broth containing appropriate concentration of antibiotic. The culture was shaken overnight at 37°C.

An aliquot of 1.5 ml overnight culture was transferred into a microfuge tube and spun down at 5000 × g for 5 minutes. The supernatant was discarded while the cell pellet was resuspended in 100 µl ice-cold solution I (Appendix 4) and vortexed vigorously. An aliquot of 200 µl freshly prepared alkaline lysis solution II (Appendix 4) was then added into the cell suspension and the content was mixed by inverting gently for 5 times. After incubation for 10 minutes on ice, 150 µl of ice-cold solution III (Appendix 4) was added into the bacterial lysate and mixed well by inverting 10 times. The tube was kept on ice for additional 10 minutes. The bacterial cell lysate was centrifuged at 10,000 × g for 10 minutes at 4°C. The resultant supernatant was transferred into a fresh microfuge tube. The DNA was extracted with equal volume of phenol, phenol:chloroform and chloroform respectively at 4°C.

Plasmid DNA was precipitated from the supernatant by adding 2 volumes of ice-cold absolute ethanol and 1/10 volume of 3 M sodium acetate (pH 4.8). DNA was pelleted at 12,000 × g for 10 minutes at 4°C followed by washing with 70% alcohol. The dried DNA pellet was dissolved in 50 µl of TE buffer (pH 8.0) containing 20 µg/ml RNase. The DNA solution was store at - 20°C or analyzed by agarose gel electrophoresis.

3.2.4 Construction of recombinant HSP20 expression vector

3.2.4.1 PCR amplification of *hsp20* gene fragment

H. pylori NCTC 11637 genomic DNA was used as the template. Primers were designed according to HslV of *H. pylori* genomic sequence (Tomb *et al*., 1997). The forward primer used was 5'-AAAGGATCCGTTTGAAGCGACGACG-3' while the reverse primer used was 5'-AAAGGATCCTTAAAGCTCCAAAATTTTAATATT-3'. Two BamHI restriction sites (underlined) were introduced in both 5' and 3' ends for insertion into the expression vector downstream of His-tag. The PCR amplification condition was set as denaturation at 94°C for 5 minutes followed by 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds and with additional extension at 72°C for 10 minutes. The PCR cycles were carried out on GeneAmp PCR system 2400 (Perkin Elmer). The PCR amplified gene fragment obtained was extracted by PCR purification kit (Qiagen).

3.2.4.2 Ligation of *hsp20* gene with expression vector pET16b

pET16b expression vector (Novagen) in *E. coli* was used for the construction of *hsp20* gene expression (Figures 3.1 & 3.2). Both purified PCR product of *hsp20* gene fragment and pET16b plasmid DNA were digested with BamHI restriction enzyme (Promega) and extracted by agarose gel extraction kit (Qiagen). T4 DNA ligase (Promega) was used to ligate the digested plasmid DNA and *hsp20* gene fragment at various molar ratios (vector: gene fragment =1:3, 1:5 & 1:10) at 16°C overnight. The ligated products were transformed into *E. coli* Top10 competent cells according to the protocol as described in section **3.2.2.2**.

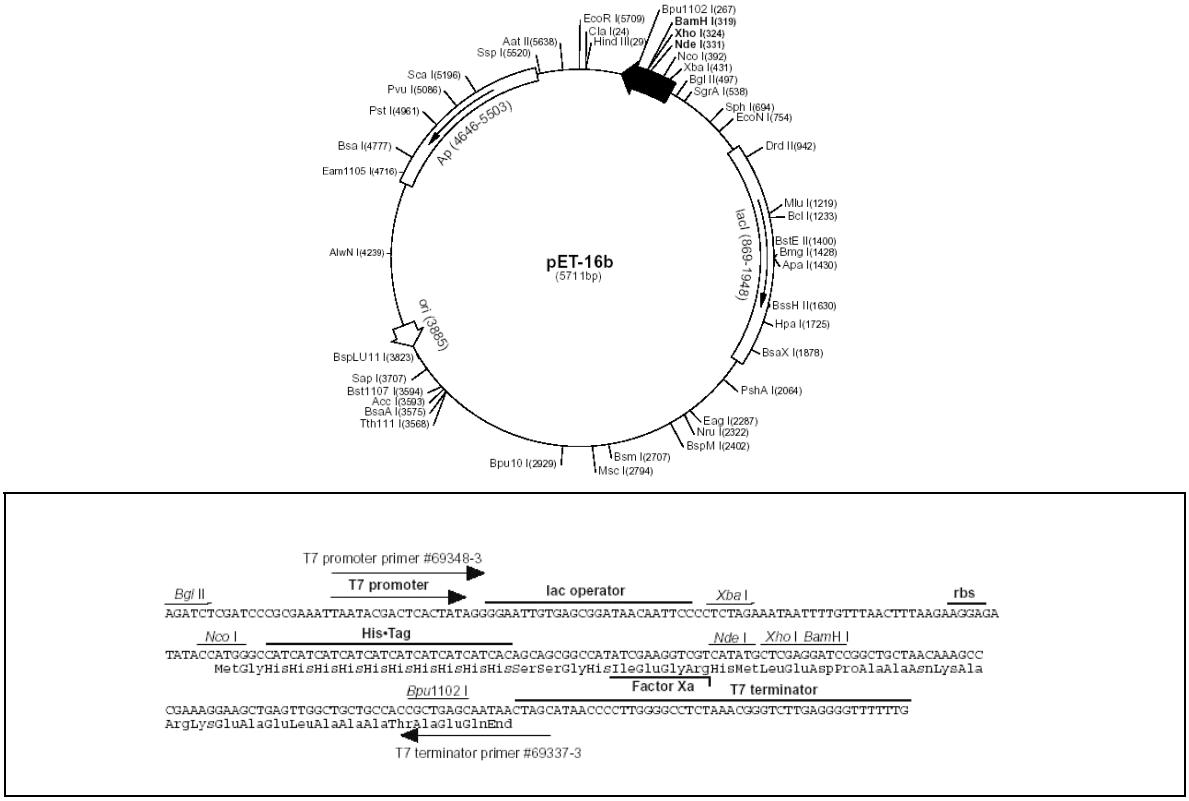


Figure 3.1 Physical map of expression vector pET16b (Novagen)

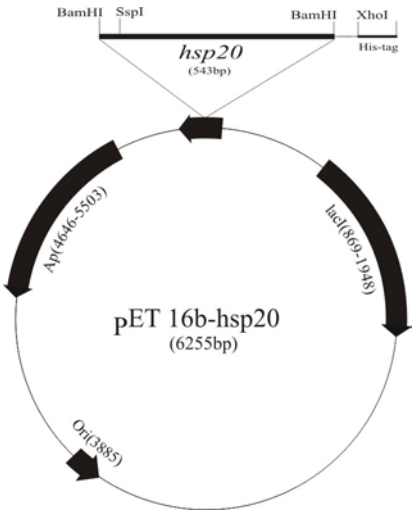


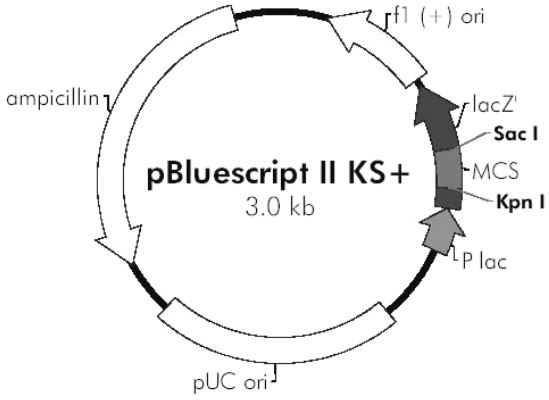
Figure 3.2 Diagrammatical representation of the construction of pET16–*hsp20* recombinant expression vector

3.2.4.3 Identification of pET16b-*hsp20* recombinant plasmid

The procedure for identification of recombinant plasmid was carried out as described by Sambrook *et al*. (1989). Single colony of transformed bacteria on LB-ampicilin agar plate was transferred into 5 ml of LB broth containing 50 μg/ml of ampicilin (Sigma). The inoculated culture was shaken at 37°C overnight. The plasmid was extracted according to the protocol as described in section **3.2.3** and digested with BamHI to screen for the presence of *hsp20* gene fragment in pET16b vector. XhoI and SspI restriction enzymes were used to digest so as to determine *hsp20* gene orientation in the vector. The correct pET16b-*hsp20* recombinant plasmid was sequenced using BigDye ™ Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer) in ABI 100 model 377 DNA sequencer (Perkin Elmer).

3.2.5 Construction of *hsp20::aphA* gene-targeting vector

The cloning vector used in the construction was pBluescript SK (+) (Stratagene, Figure 3.3). *aphA* gene (Kanamycin resistant gene) was inserted into *hsp20* (HP0515) that was flanked by HP0513 – HP0517.



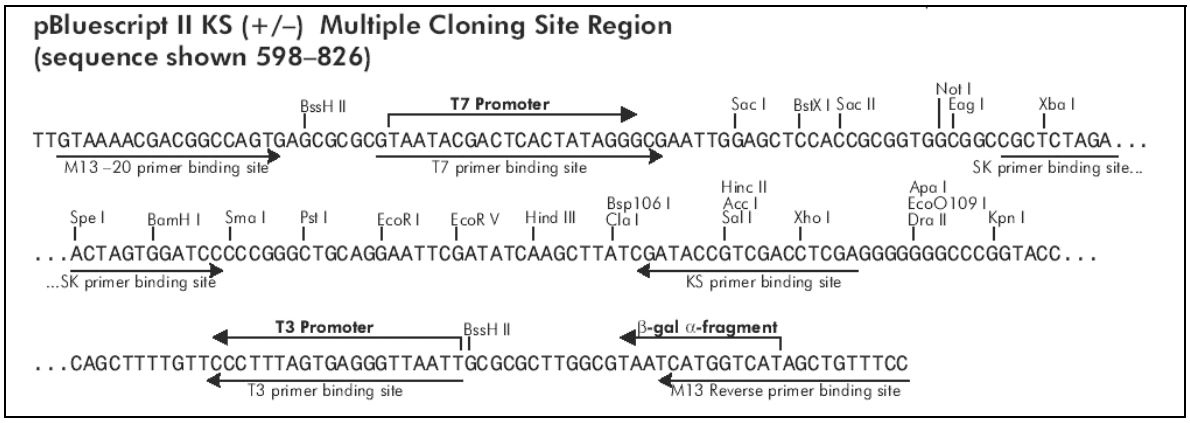


Figure 3.3 Physical map of *E. coli* cloning vector pBluescript SK(+) (Stratagene)

3.2.5.1 PCR amplification of flanking sequences of targeting vector

Genomic DNA of *H. pylori* SS1 was used as the template for amplification. Four primers (KO1, 2, 3 & 4, Table 3.1) were designed according to the known *H. pylori* 26695 DNA sequences (Tomb *et al*., 1997) for the amplification of two flanking DNA sequences. The 5' flanking DNA fragment of 2700 bp was amplified using KO1 & 2 primers. This fragment included HP0513, HP0514 and the initial 270 bp of HP0515 of *H. pylori* 26695 DNA sequences (Tomb *et al*., 1997) with BamHI and PstI sites at 5' and 3' ends respectively. The 3' fragment was 2549 bp long including the rest of the 273 bp of HP0515, HP0516 and HP0517 (Tomb *et al*., 1997) with SalI and ApaI sites at 5' and 3' ends respectively. This fragment was amplified using KO3 & 4 primers (Figure 3.4 and Table 3.1). PCR amplification was performed by an initial denaturation at 94°C for 5 minutes followed by 94°C, 30 seconds, 50°C or 55°C, 1 minute, 72°C, 1 minute and 30 seconds for 30 cycles with additional extension at 72°C for 10 minutes. The PCR cycles were carried out on GeneAmp PCR system 2400 and the PCR amplified flanking DNA fragments were extracted by PCR purification kit.

Table 3.1 Primers used for amplification of two flanking DNA fragments of *hsp20* (HP0515)

Name	Sequences (5' – 3')	Length of fragment	Tm (°C)
KO1	CGGGATCCATGAACGGACATTTTATCGGTT (BamHI)	2700 bp	55
KO2	AACTGCAGCCATTCTTTACTGAAATCCACC (PstI)		
KO3	ACGCGTCGACCGCAAAGATAAGTATTTACGC (SalI)	2549 bp	50
KO4	TCCGGGCCCTCAATCCCTATTCCTTCTATGGA (ApaI)		

Restriction enzyme sites were underlined.

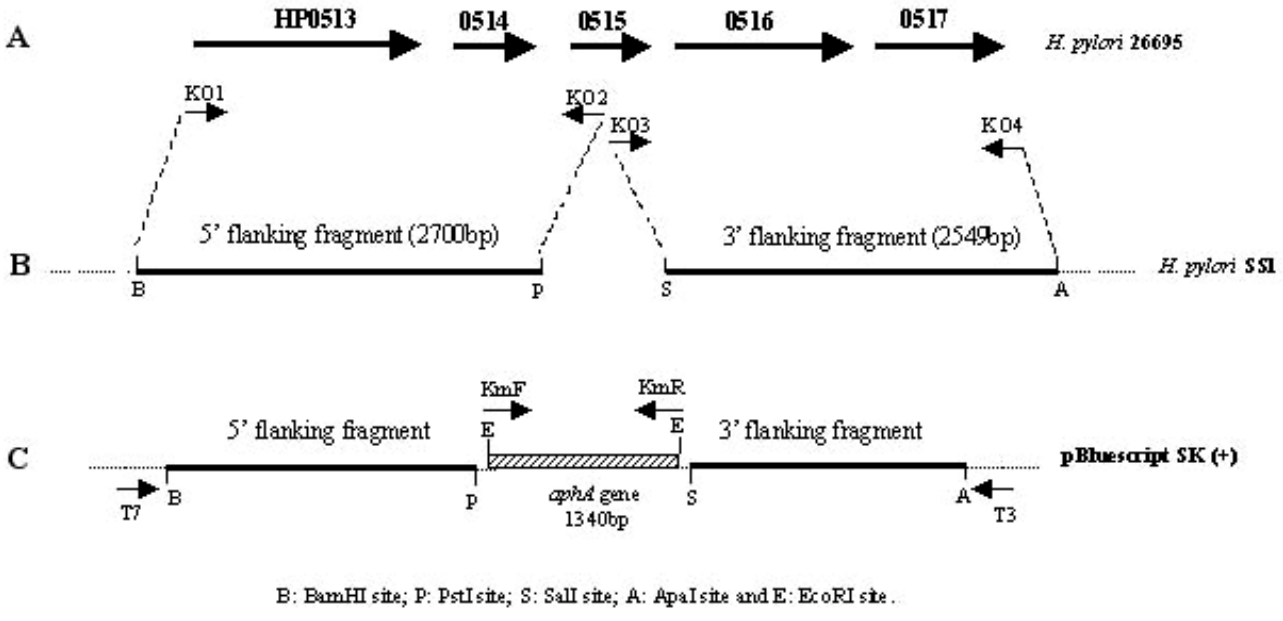


Figure 3.4 Schematic construction of *hsp20::aphA* gene-targeting vector

A, the location of genes in genome of *H. pylori* 26695; four primers were designed based on the known sequences; B, two flanking DNA fragemnts were amplified using *H. pylori* SS1 genomic DNA as template; C, the insertion of two flanking fragments and *aphA* gene in pBluescript SK (+); the full length of targeting vector is 9589bp.

3.2.5.2 The selective marker – Kanamycin resistant gene (*aphA*)

Kanamycin resistant gene (*aphA* cassette with its own promoter region, 1340 bp) was excised from plasmid pILL600 by EcoRI (Promega) restriction enzyme digestion. pILL600 plasmid was kindly provided by A. Labigne (Pasteur Institute, Paris, France) (Ferrero *et al*., 1992). The *aphA* gene obtained was purified by gel extraction kit.

3.2.5.3 Ligation of two flanking DNA fragments and *aphA* gene

Cloning vector pBluescript-SK(+) (Stratagene, Figure 3.3) was used in the construction of *hsp20* gene targeting vector. Firstly, two flanking DNA fragments (5' & 3') was inserted into pBluescript SK(+) plamid at the corresponding sites BamHI & PstI and SalI & ApaI for 5' and 3' flanking fragments, respectively (recombinant plasmid: pBS-5' & pBS-3'). *aphA* gene were inserted into pBluescript SK(+) plamid at EcoRI site (recombinant plasmid: pBS-*aphA*). The two flanking DNA fragments were then excised from the two individual recombinant plasmids (pBS-5' & pBS-3') and inserted into pBS-*aph*A for construction of recombinant targeting vector (pBS-5'-*aphA*-3', Figure 3.4). The procedure for ligation and transformation were performed as described in section **3.2.4.2** and **3.2.2.2,** respectively.

3.2.5.4 Identification of *hsp20::aphA* gene-targeting vector

The procedure for identification of all recombinant plasmids was carried out as described in section **3.2.4.3**. The presence of respective DNA fragments in recombinant plasmids was screened using corresponding restriction enzyme digestions (5' fragment: BamHI & PstI; 3' fragment: SalI & ApaI; *aphA* gene: EcoRI). The 3 recombinant

plasmids containing 3 digestible corresponding fragments were assumed to have correct insertion and then sequenced using BigDye ™ Terminator Cycle Sequencing Ready Reaction Kit in ABI 100 model 377 DNA sequencer.

3.2.6 Transformation of *H. pylori* with the gene-targeting vector

The protocol for transformation of *H. pylori* with plasmid DNA was carried out as described by Heuermann & Haas (1998). Briefly, 2- 3 days old *H. pylori* SS1 cells grown were harvested from CBA plates and suspended in BHI broth to obtain a cell density of 10^9 - 10^{10} CFU/ml. An aliquot of 200 μl *H. pylori* cell suspension was then transferred into a sterilize microfuge tube and 1 μg targeting plasmid DNA (pBS-5-*aphA*-3') was added to the *H. pylori* cells. The mixture (200 μl) was spotted on the surface of CBA plate (without antibiotics) and incubated at 37°C with 5% CO_2 for 4 hours before spreading the bacterial mixture over the whole plate. After incubation at 37°C with 5% CO_2 for 16 - 18 hours, the lawn of cells was transferred onto a fresh CBA plate containing 25 μg/ml kanamycin using inoculation loop and further incubated at 37°C with 5% CO_2 for 3 – 4 days. The colonies that grown on the kanamycin containing CBA plate were selected and sub-cultured for a few rounds to purify *hsp20::aphA* transformed *H. pylori* mutants. The purified *hsp20*-isogenic *H. pylori* mutant was preserved in BHI broth medium supplemented with 10% horse serum and 20% glycerol and stored at -80°C for further analysis.

3.2.7 Identification of *hsp20*-isogenic *H. pylori*

The identification of *hsp20*-isogenic *H. pylori* was performed as described by Goodwin *et al*. (1998). PCR amplification (using different primers, Table 3.2) and Southern blot hybridization (using different probes, *aphA* gene fragment & pBluescript plasmid DNA) were employed to identify the mutant. Western blotting was used to analyze the expression of HSP20 in the mutant.

Table 3.2 Primers used for the identification of *aphA* insertion in the *H. pylori* genome

Name	Sequences (5' – 3')	Length of DNA fragment (bp)	Tm (°C)
HSPF	CGGGATCCATGTTTGAAGCGACGACGATTTTAGGC	1883	58
HSPR	CGGGATCCTTAAAGCTCCAAAATTTTAATATTCGTG		
KmF	CGGGATCCGATAAACCCAGCGAACCATTTGAG	1340	55
KmR	CGGGATCCAAGCTTTTTAGACATCTAAATCTAGGT		
HSPF	CGGGATCCATGTTTGAAGCGACGACGATTTTAGGC	1612	52
KmR	CGGGATCCAAGCTTTTTAGACATCTAAATCTAGGT		
KO3	ACGCGTCGACCGCAAAGATAAGTATTTACGC	2614	52
T7	GTAATACGACTCACTATAGGGC		

Km: Kanamycin resistant gene (*aphA*).

3.2.7.1 PCR amplification

The genomic DNA of *hsp20*-isogenic *H. pylori* was extracted according to the protocol as described in section 3.2.1. Different pairs of primers (Table 3.2) were used in the amplification to identify the insertion of *aphA* gene in the genome. One pair of

primers (forward: HSPF & reverse: HSPR) was based on *hsp20* gene sequences of *H. pylori* 26695 (Tomb *et al*., 1997) while another pair of primers (5' primer: KmF & 3' primer: KmR) was based on the sequences of *aphA* gene (Ferrero *et al*., 1992). T7 promoter primer was based on the sequences of pBluescript SK(+) DNA. The genomic DNA of wild type *H. pylori* was used as the negative control while targeting vector (pBS-5-*aphA*-3') was used as the positive control. The PCR cycles were carried out on GeneAmp PCR system 2400 according to the conditions described in section **3.2.5.1**.

3.2.7.2 Southern blotting hybridization

Southern blot hybridization was carried out using ECL Direct™ Nucleic Acid Labeling and Detection System (Amersham BioScience). The procedure was performed according to the instruction provided by the manufacturer. In brief, 5 μg genomic DNA was digested with EcoRI restriction enzyme overnight at 37°C. The digested genomic DNA was loaded in the wells of agarose gel (6× loading buffer, Appendix 1) and ran with 1× TAE buffer (Appendix 17) at 50 volts for 4 – 5 hours. The image of the gel was recorded using camera under UV illuminator. The gel with DNA samples was treated with depurination (250mM HCl) and denaturation (1.5M NaCl & 0.5M NaOH) solutions for 10 and 25 minutes respectively. The gel was kept in neutralizing solution with agitation for 30 minutes on a shaker with one change of neutralizing solution and further agitated for 15 minutes before blotting. The same size of Hybond N+ nylon membrane (Amersham BioScience) was soaked in 20× SSC for at least 10 minutes prior to use. Three sheets of 3M paper cut to the same size as the nylon membrane were pre-wetted with 10× SSC. The capillary blotting was assembled as shown in Figure 3.5. The blotted

membrane after overnight blotting was rinsed with 6× SSC and baked at 80°C for 2 hours. Horseradish peroxidase (HRP) conjugate specific probes were prepared according to the instruction provided by manufacturer at concentration of 100 ng/10 µl. Hybridization was carried out overnight in hybridization glass tubes with gentle rotation in an hybridization oven (Amersham BioScience) at 42°C. Two stringent washes were applied at 42°C. The hybridization signal was generated with the addition of detection buffers (Amersham BioScience) before exposing onto the autoradiography film in cassette in the dark at various time intervals. The genomic DNA of wild type *H. pylori* was used as the negative control. *hsp20* gene-targeting vector (pBS-5-*aphA*-3') (10 ng) was used as the positive control.

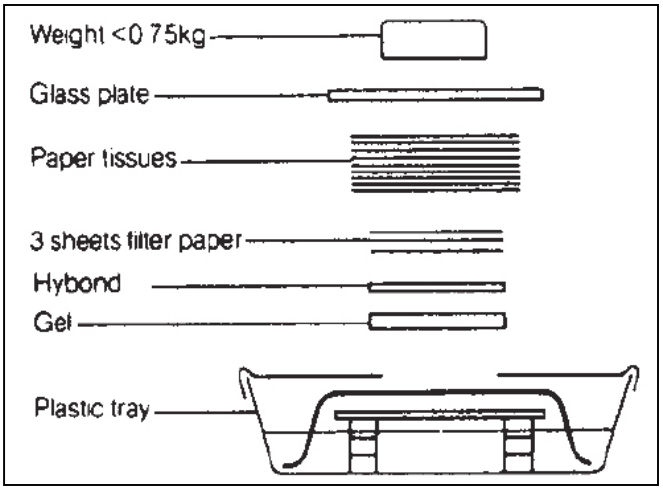


Figure 3.5 Capillary blotting assembly (Amersham)

3.3 Proteomic analysis

3.3.1 Bio-rad protein assay

The protocol was carried out according to the manufacturer's instruction (Bio-rad). The dye reaction solution was prepared by mixing 1 part of concentrated dye solution (acidic solution of Coomassie Brilliant Blue G-250) with 4 part of deionized water and filtered through Whatman filter paper. In this study the reaction volume of dye was minimized to 1 ml. BSA protein standards were prepared (0.2 – 1 mg/ml). Mixed each 20 µl protein standard and sample with 1 ml diluted dye reagent and incubated at room temperature for at least 5 minutes. The protein concentration of the sample was calculated based on the standard curve plotted by protein standards against OD_{595}.

When measuring the concentration of proteins prepared by lysis buffer [lysis buffer: 8 M urea, 4% CHAPS, 40mM Tris-Cl pH 8.8, protease inhibitor cocktail (Roche) and freshly prepared 50 mM DTT], a modified protein assay was used in which an additional 10 µl of lysis buffer and 10 µl of 0.1 M HCl were added into the reactions for correction.

3.3.2 SDS-PAGE

SDS-PAGE analyses were performed according to the method as described by Bollag *et al*. (1996).

3.3.2.1 Preparation of gel

A 1mm slab gel consists of stacking and separation gels is prepared according to the method as described by Bollag *et al*. (1996). The final concentration of acrylamide used

in stacking gel is 5% and separation gel ranges from 10 – 15%. The recipe used for gel preparation is listed in Appendix 15 (Bollag *et al*., 1996).

3.3.2.2 Sample preparation and gel running

A desired amount of protein was mixed with 2× SDS sample buffer (100 mM Tris-HCl pH 6.8; 200 mM DTT; 4% SDS; 0.2% bromaphenol blue; 20% glycerol), denatured by boiling for 5 minutes and loaded into the wells of the slab gel placed in a mini-PROTEAN 3 or PROTEAN II vertical electrophoresis system (Bio-rad). The gel was ran using 1× Tris-glycine buffer (25 mM Tris base; 250 mM glycine; 0.1% SDS; pH 8.3) at room temperature for 1 –2 hours at 100 volts with PowerPac basic (Bio-rad) till the front line of dye reached the bottom of the gel.

3.3.2.3 Gel staining and visualization of protein bands

The gel with protein bands was stained by coomassie blue (R-250) or silver nitrate. The protocols for staining were carried out as described by Bollag *et al*. (1996).

In coomassie blue staining, the gel was soaked in 0.1% coomassie blue R-250 solution [0.1% (W/V) coomassie blues R-250; 50% methanol; 5% acetic acid and 45% distilled water] with gentle shaking for 2 hours. The gel was washed with destaining solution (40% methanol; 10% acetic acid and 50% distilled water) until the background became clear.

In silver staining, the gel was fixed with fixation solution (50% methanol; 5% acetic acid and 45% distilled water) for 30 minutes and rinsed in distilled water for 1 hour with 3 changes. The fixed gel was sensitized with 0.02% (W/V) sodium thiosulfate (Sigma) at

room temperature for 2 minutes and rinsed in distilled water twice at 1 minute per washing. The gel was then stained with 0.1% (W/V) silver nitrate (Sigma) for additional 20 minutes in the dark and rinsed twice with distilled water. The stained gel was developed with solution [2% (W/V) sodium carbonate; 0.04% (V/V) 37% formaldehyde] until the desired intensity was achieved. The reaction was then stopped with 5% (W/V) acetic acid solution.

The protein bands on the gels were scanned with GS-710 calibrated Densitometer (Bio-rad) and analyzed using Quantity One program (Bio-rad).

3.3.3 Two dimensional gel electrophoresis (2-DE)

Processing steps of 2-DE were carried out according to the manufacturer's instruction as described by Berkelman & Stenstedt (1998). There are two main steps included in the first dimension of 2-DE, these are rehydration and focusing.

In the step of rehydration, the required amount of protein sample (e.g. 20 – 30 μg for a 7cm IPG strip; 80 – 500 μg for a 17cm IPG strip) was mixed with rehydration buffer. The rehydration buffer comprises 8 M urea, 4% CHAPS (Roche), 10 mM DTT and 0.2% (W/V) Biolytes 3/10 (Bio-rad). DTT and Biolytes were added freshly. The mixture of protein sample in rehydration buffer was loaded into the IPG strip holder (Bio-rad) that contained the IPG strip of appropriate pH range and size (Bio-rad) before the addition of mineral oil. The process of rehydration was carried out at room temperature at 50 volts for 10 - 12 hours in PROTEAN IEF System (Bio-rad).

At the end of the rehydration step, the protein sample absorbed in the IPG strip was then proceeded to iso-electric focusing (IEF). The IEF was carried out in PROTEAN IEF

System under different conditions according to the size of IPG strip (Table 3.3). When the process of IEF was completed, the IPG strip was removed from the strip holder and stored at –20°C until use or directly run on the second dimension SDS-PAGE.

Before running SDS-PAGE, the IPG strip was washed with equilibration buffer/DTT followed by washing with equilibration buffer/iodoacetamide (IAA) once, each for 15 minutes. The equilibration buffer used consists of 6 M urea, 0.375 M Tris-Cl (pH 8.8), 2% SDS, 20% glycerol and 2% (W/V) DTT or 2.5% (W/V) IAA (DTT or IAA added freshly).

SDS-PAGE was run on the vertical electrophoresis system: Mini-PROTEAN 3 or PROTEAN xi/XL Vertical Electrophoresis Cells (Bio-rad) depending on the length of the strip. The protocols for SDS-PAGE were the same as described in section **3.3.2**.

Table 3.3 Optimal IEF conditions for different sizes of IPG strips

Steps	7 cm IPG strip	11 cm IPG strip	17 cm IPG strip
1	300 volts 1hour	300 volts 1 hour	300 volts 1 hour
2	1000 volts 1 hour	1000 volts 1 hour	1000 volts 1 hour
3	3000 volts 1 hour	3000 volts 1 hour	3000 volts 1 hour
4	4000 volts 12000 volthours	5000 volts 35000 volthours	6000 volts 60000 volthours
5	500 volts hold	500 volts hold	500 volts hold

3.3.4 Protein identification by mass spectrometry (MS)

The protein sample (spot from 2D gel and band from SDS-PAGE) of interest as observable by coomassie blue staining was digested with modified sequencing grade trypsin (Promega) according to the protocol of in-gel trypsin digestion as suggested by

Shevchenko *et al*. (1996). All the reagents used for digestion must be HPLC grade. The target protein band was excised from the coomassie blue stained gel and was grinded into small pieces in a micro-centrifuge tube. Aliquot of 100 µl of 50 mM ammonium bicarbonate (NH_4HCO_3)/50% (V/V) acetonitrile (MERCK) was added to immerse the gel pieces, votexed and let stand for 5 minutes. The solution was removed by micropipette and the same step was repeated for 2 - 3 times. The bleached gel pieces were then treated with 50 µl actetonitrile for 5 minutes. After removing the acetonitrile, the gel pieces were dried in a vacuum-speed (Heto).

Aliquot of 50 µl of 10 mM DTT in 100 mM ammonium bicarbonate was added to reduce the disulfide bonds at 57°C for 1 hour. The excess DTT solution was removed then alkalized by adding 55 mM iodoacetamide (IAA) in 100 mM ammonium bicarbonate. The reaction was carried out at room temperature in the dark for 1 hour. After alkylation, the gel pieces were washed with 100 µl of 100 mM ammonium bicarbonate for 5 minutes and dehydrated with the same volume of acetonitrile. The same operation was repeated. The dried gel pieces were re-swelled in 15 – 30 µl digestion solution (12.5 ng/µl trypsin in 50 mM ammonium bicarbonate) and incubated at 4°C for 30 – 60 minutes. The excess trypsin solution was removed and 15 µl of 50 mM ammonium bicarbonate was added. The digestion was continued overnight at 37°C.

The digested gel pieces in micro-centrifuge tube were spun at 6000 × g for 5 minutes and the supernatant was transferred into a fresh micro-centrifuge tube. The gel pieces were further treated with 10 – 20 µl of 20 mM ammonium bicarbonate and 5% formic acid in 50% aqueous acetonitrile, 5 minutes for each treatment. The supernatant collected were combined and dried to the desired volume by vacuum speed.

The protein sample obtained was sent to the Proteins and Proteomics Centre, National University of Singapore for protein identification by mass spectrometry. Matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) (Voyager-DE™ STR Biospectrometry™) (Applied Biosystem) based on the mass fingerprinting of trypsin-digested peptides was used for the identification of protein isolated in the later study (e.g. CO-IP). Quadrupole time of flight mass spectrometry (Q-TOF MS) (Micromass Q-Tof Tandem Mass Spectrometer) (Applied Biosystem) that is based on both mass fingerprinting of peptides and sequencing of representative peptide was used for the identification of recombinant HSP20.

3.4 Immunological analysis

3.4.1 Enzyme-linked immunosorbent assay (ELISA)

Indirect ELISA analysis used was as described by Delves (1995). Briefly, the required antigen was diluted with carbonate coating buffer (pH 9.6, Appendix 6) to the concentration of 1 – 10 μg/ml. Aliquot of 100 μl of the diluted antigen was coated onto each well of the microtitre plate (NUNC) and incubated overnight at 4°C. The coated plate was washed three times with PBS-T buffer (Appendix 12) and blocked with 5% (W/V) BSA–PBS-T at 37°C for 2 hours. Aliquots of 100 μl primary antibody PBS-T diluents (ranging from 1:100 – 500) were added in triplicates and incubated at 37°C for additional 2 hours. Amount of 100 μl of diluted HRP conjugated secondary antibody (DAKO, 1:2000) was added to each well and incubated for 2 hours. The plate was then washed with PBS-T for 3 × 5mins. The substrate, 0.4% O-phenylenediamine dihydrochloride (OPD) was added for the enzymatic reaction (substrate buffer) before it

was stopped by the addition of stopping buffer (2M H_2SO_4). The result was read at OD_{492} in a Labsystems Multiskan Ascent microtitre plate reader (Vantaa).

3.4.2 Western blotting

Antibodies from different sources [antibody against rHSP20 (raised in this study), anti-serum against *H. pylori* HSP60 (kindly provided by Lund University of Sweden), anti-serum against rCagA (prepared for this study), antibody against *H. pylori* (DAKO), goat anti-rabbit HRP conjugate (DAKO) and goat anti-mouse HRP conjugate (DAKO)] were used to detect the presence of specific protein in the protein mixture. The protocols for Western blotting analysis were carried out as described by Sambrook *et al*. (1989).

3.4.2.1 SDS-PAGE of protein samples

The required amount of protein samples (15 – 30 μg) were denatured by mixing it with 2× SDS sample buffer and boiled at 100°C for 5 minutes. The samples were then run on SDS-PAGE gel according to the protocol as described in section **3.3.2**.

3.4.2.2 Blotting

PVDF membrane (Millipore) that was of the same size as the gel was activated by soaking in methanol for 20 seconds before immersing in transfer buffer (48 mM Tris base, 39 mM glycine, 0.037% SDS, 20% methanol) until use. Eight pieces of Whatman filter paper of the same size as the gel were soaked in the transfer buffer. A stack of "sandwich" consisting of 4 sheets of filter paper, PVDF membrane, SDS-PAGE gel and another 4 sheets of filter paper was assembled in the order as described and placed on the

blotting cell (Bio-rad). The blotting process was carried out at room temperature for 1 hour at 150 mA (Bio-rad). When the blotting was complete, the membrane was transferred into PBST buffer. The gel was then stained with coomassie blue solution to examine if the blotting was complete.

3.4.2.3 Incubation with primary and secondary antibodies

The blotted membrane was rinsed with PBS-T buffer three times for 5 minutes each and blocked with 5% (W/V) skim milk–PBS-T at 37°C for 2 hours or at 4°C overnight. After blocking, the membrane was washed three times with PBS-T to remove the skim milk. The membrane was incubated with primary antibody and secondary antibody – HRP conjugate consecutively at 37°C for 2 hours for each reaction. There were three washing with PBS-T buffer of 5 minutes each in between each incubation period. The antibody-conjugated membrane was stained with 4-chloro-napthol (4-CN) as a substrate or by using ECL detection kit (Amersham Biosciences) according to the instructions provided by manufacturer.

3.4.3 Flow cytometry

This method was used to analyze the fluorescence dye labeled antigens on the cells surface. The protocol for fluorescence labeling was performed as proposed by Blom *et al*. (2001). The 3 days old *H. pylori* cells pellet was suspended in appropriate volume of ice-cold 0.1% (W/V) BSA-PBS buffer. The appropriately diluted primary antibody (rabbit anti-*H. pylori*, DAKO, 1:20000 diluted) was added. The mixture of cells and antibody was incubated on ice for 30 minutes with occasional inversion. The cells were spun down

at 5000 × g for 5 minutes and washed twice with 0.1% BSA-PBS buffer at 4°C. Similarly, the diluted secondary antibody FITC-conjugate (goat anti-rabbit IgG FITC conjugate, Sigma, 1:500 diluted) was added into the primary antibody labeled cell suspension and incubated for additional 30 minutes on ice. The antibody labeled cells were washed twice with 0.1% BSA-PBS at 4°C and fixed with 0.5% (V/V) paraformaldehyde-PBS buffer before running on Coulter Epics Elite ESP flow cytometer (Spectron Corp). The data obtained were analyzed by program WinMDI Version 2.8.

3.5 Preparation of different *Helicobacter pylori* sub-cellular fractions

3.5.1 Total protein (TP)

H. pylori cells were lysed according to the method as described by Berkelman & Stenstedt (1998). A 3-day old *H. pylori* culture was washed three times with ice-cold PBS and resuspended in 300 – 400 μl of lysis buffer per 10^9 cells [lysis buffer: 8 M urea, 4% CHAPS, 40mM Tris-Cl pH 8.8, protease inhibitor cocktail (Roche) and freshly prepared 50 mM DTT]. The cell suspension was left on ice for 1 hour, vortexed occasionally. DNase (20U) and RNase (20U) were added into the cell suspension and left on ice for additional 10 minutes before centrifuging at 10,000 × g for 10 minutes at 4°C. The resultant supernatant was transferred into fresh micro-centrifuge tube and stored at -80°C until use. The lysed fraction consists primarily of total protein of *H. pylori.* The protein concentration was measured by using modified Bio-rad protein assay as described in section **3.3.1**.

3.5.2 Acid glycine extract (AGE)

The protocol for preparation of AGE was carried out according to the modified method as described by Ho & Jiang (1995). In brief, 3-day old *H. pylori* culture grown in BHI broth or chocolate blood agar plate (10^8/ml) was harvested and lysed by using 0.2 M acid glycine (pH 2.2) with constant stirring for 30 minutes at 4°C. The resultant cell lysate was then centrifuged at 10,000 × g for 15 minutes at 4°C. Supernatant collected was dialysed against PBS buffer at 4°C overnight in dialysis tubing (Gibco). The AGE mainly comprises cell membrane and membrane associated proteins. The protein concentration of the dialysate was measured using Bio-rad protein assay.

3.5.3. Outer membrane protein (OMP)

OMP of *H. pylori* was isolated according to the method as described by Ascencio *et al*., 1998). Harvested 3-day old *H. pylori* cells (10^8/ml) were broken by sonication (30s × 3) in a Soniprep sonicator (Sanyo). Unbroken cells were removed by centrifugation at 5000 × g for 30 minutes at 4°C. The supernatant was further centrifuged at 20,000 × g for 90 minutes at 4°C. The pellet was suspended in 150 µl of distilled water. Samples were treated with 8 volumes of 2% sodium N-laurylsarcosine (Sigma) for 1 hour at room temperature. The insoluble OMP was pelleted twice by centrifugation at 20,000 × g for 60 minutes at 4°C and washed twice with 2 ml deionized water to remove the excess detergent. Finally, the pellet was resuspended in 50 µl of distilled water. The protein concentration was measured as described in section **3.3.1**.

3.5.4 Cytoplamic protein (CP)

CP protein fraction of *H. pylori* cells was extracted according to Chmiela *et al*. (1996). Briefly, a 3-day old *H. pylori* culture was harvested and the cell pellet was resuspended in appropriate volume of PBS buffer (to give ~ 10^9cells/ml), followed by sonication for 3 cycles of 30 seconds each. The cell suspension was centrifuged at 20,000 × g for 90 minutes at 4°C. The resultant supernatant was collected and sterilized by filtration through 0.2 μm filter (Sartorius). The protein concentration was measured by Bio-rad protein assay as described in section **3.3.1**.

3.6 Expression and purification of recombinant HSP20 (rHSP20)

3.6.1 Induced expression of recombinant protein (rHSP20)

The recombinant protein (rHSP20) was induced as recommended by the manufacturer (pET System Manual, Novagen, 2001). The constructed recombinant expression vector pET16b-*hsp20* was transformed into *E. coli* expression strain BL-21 (DE3). To optimize the conditions for induction, a single colony was picked from the plate of transformed BL-21 bacteria and inoculated into 50 ml of LB medium supplemented with 50 μg/ml ampicilin (Sigma). The culture was shaken vigorously (200 rpm) at 37°C until OD_{600} of 0.5 was reached. IPTG at 0.4 mM was added into the medium. The induced cells were collected at different time points over 4 hours of incubation. The harvested cells were then lysed with 2 × SDS sample buffer and subjected to SDS-PAGE analysis as described in section **3.3.2**.

The large scale of expression of recombinant protein was carried out in 1L LB medium according to the optimized conditions for induction.

3.6.2 Purification of rHSP20 by affinity chromatography

Upon achieving the maximum expression, *E. coli* cells were harvested at 5000 × g for 5 min at 4°C and resuspended in 20 mM phosphate saline buffer (PSB, Appendix 11). The cell suspension (10^8/ml) was subjected to sonication for 10×15s at 5 MHz amplitude in a Soniprep 150 sonicator (Sanyo) on ice-bath. The whole cell lysate was centrifuged at 10,000 × g for 10 minutes at 4°C. Both supernatant & pellet were used for SDS-PAGE analysis to determine the location of expressed recombinant protein. The results showed that the expressed rHSP20 was found to exist as inclusion body in host cells. Therefore, the inclusion body collected was further washed twice with ice-cold PSB buffer and dissolved overnight in 6M urea - PSB at 4°C. The dissolved content was centrifuged at 12,000 × g for 15 minutes at 4°C and filtered through 0.45 μm syringe filter (Millipore). Purification of rHSP20 was carried out by using affinity chromatography through nickel chelating column (Amersham Biosciences) under denatured conditions according to manufacturer's instructions. The buffers (charge, binding, wash, elution and stripping buffers) used for affinity chromatography are included in Appendix 3. The targeted protein in nickel column was eluted in ascending gradient with imidazole ranging from 0.3 – 1M. Purified recombinant HSP20 (rHSP20) was refolded through dialysis against 20 mM PSB at 4°C. All fractions collected throughout purification procedure were subjected to SDS-PAGE analysis and protein concentration was measured by Bio-rad protein assay (section **3.3.1**). The purified rHSP20 was identified by Q-TOF mass spectrometry as described in section **3.3.4**

3.7 Raising antibody against rHSP20 in rabbits

3.7.1 Immunization procedure of rabbit with rHSP20

Antibody against rHSP20 was raised in rabbits and immunization procedure was followed as recommended by Coligan *et al*. (2001). The study was approved by the Animal Experimental Ethic Committee, National University of Singapore. The mixture of purified rHSP20 (two doses: 120 μg & 150 μg) and Freud's incomplete adjuvant (Sigma, USA) was injected into New Zealand white rabbits (~ 1.2 kg) intramuscularly. Two boosters were given at the 5th week after the 1st immunization and 3 weeks after the 2nd immunization. Blood was drawn from the rabbits at the time of immunizations and every 7 days after the 2nd booster (Table 3.4). Serum was separated from whole blood and antibody titer was assayed by indirect ELISA method using 0.5 μg purified rHSP20 as antigen.

Table 3.4 Immunization procedures for raising antibodies against rHSP20 in rabbits

	1st immunization	**2nd immunization (1st booster)**	**3rd immunization (2nd booster)**
Time	1st week	5th week	7th week
Dosages of Ag	120 and150 μg	120 and150 μg	120 and150 μg
Adjuvant	FIA	FIA	FIA
Amount of blood drawn	10 -15 ml	10 – 15ml	10 ml at each time at weekly interval upon 12th weeks
Method of Ab detection	ELISA	ELISA	ELISA

FIA: Freud's incomplete adjuvant; Ab: antibody; Ag: antigen; ELISA: enzyme linked immuno-absorbance assay.

3.7.2 Purification of antibody

Protein A sepharose CL-4B (Amersham Biosciences) affinity column was used to purify IgG from anti-serum (Coligan *et al*., 2001) because of the specific binding between Protein A and Fc portion of IgG. The purification was carried out as described according to the manufacturer's instructions. The serum was diluted with 4 volumes of 50 mM Tris-HCl buffer (pH 7.0) and loaded onto the column. The specific IgG was eluted by 0.1 M glycine-HCl buffer pH 3.0 and neutralized with 1 M Tris-HCl pH 9.0 (50-100µl/ml fraction) immediately after elution. All buffers used must be kept at 4°C or on ice-bath during the operation. The purified IgG was analyzed by SDS-PAGE and protein concentration was measured by Bio-rad protein assay (section **3.3.1**). The antibody solution was stored at -80°C until use.

3.7.3 Characterization of antibody

Antibody obtained was characterized on its binding specificity using Western blotting on different protein fractions of *H. pylori* cells according to the method as described by (Delves, 1995). The fractions of TP, AGE, OMP and CP of *H. pylori* cells were examined. The rabbit anti-serum against *H. pylori* HSP60 (kindly provided by Prof T. Wadstrom, Lund University, Sweden) was used as an unrelated positive control in testing the localization of HSP20 by Western blotting.

3.8 Immuno-gold labeled transmission electron microscopy (TEM)

Pre-embedding labeling was employed for immuno-gold labeled TEM as described by Polak & Varndel (1984). In this experiment, purified polyclonal rabbit IgG against

rHSP20 (25 μg/ml) was used as the primary antibody while the second antibody used was 5 nm, 10 nm or 20 nm gold-Protein A conjugate (1:20 dilution, Ted Pella). *H. pylori* NCTC 11637 3-day old broth culture was harvested and washed with PBS, then fixed in 4% paraformaldehyde, 0.1% glutaraldehyde, with or without Triton X-100 (0.5%). Neutralization was done by incubating the cell pellet in 0.05 M glycine-PBS for 15 minutes and washed with PBS. The fixed cells were blocked with 0.5% (W/V) BSA-PBS buffer and then conjugated with the primary antibody at 37°C for 2 hours followed by incubating with the secondary antibody at 37°C for additional 2 hours. The immuno-gold labeled cells were further fixed in 2% glutaraldehyde at room temperature for 2 hours. The fixed immuno-gold labeled cells were processed through dehydration, infiltration and embedding in Low Viscosity Epoxy Resin (LVER) (Agar Scientific). Ultra-thin sections (70 – 100 nm) were viewed under Philips 208S electronic microscope. The 0.5% Triton X-100 (Hannah *et al*., 1998) was used to partially solubilize the cell membrane for 10 or 20 minutes during the fixation step before the addition of secondary antibodies. The pre-immune serum and the secondary antibody added alone were served as negative controls. In testing the effectiveness of solubilization of cell membrane using Triton X-100 treatment, *E. coli* cells labeled with anti-rHSP20 (25 μg/ml) were used as negative control while anti-*H. pylori* HSP60 antiserum (1:20) labeled *H. pylori* served as the internal positive control.

3.9 Detection of antibody against HSP20 in patients with gastroduodenal diseases

In this study, rHSP20 was used as the antigen to detect the presence of antibody against HSP20 in sera obtained from patients with different gastro-duodenal diseases by

ELISA as described in section **3.4.1**. A total of 57 sera samples from patients (peptic ulcer: 25 and non-ulcer dyspepsia: 32 respectively) were tested. The disease status of the patients was confirmed earlier by histological examinations and serological testing. Sera from 32 normal subjects without any gastroduodenal complaint served as negative control. All samples were randomly selected. Data obtained were analyzed statistically by Student t-test.

3.10 *In vitro* adhesion assay

The adhesion assay of *H. pylori* to human gastric carcinoma cell lines, Kato III and AGS, was performed according to the methods as described by Yamaguchi *et al*. (1996). Either wild type *H. pylori* or *hsp20*-isogenic *H. pylori* mutant was added at different ratios of bacteria to cells (bacteria : cells = 50:1, 100:1 and 200:1). Cells and bacteria were suspended in RPMI:BHI (1:1) medium and co-incubated at 37°C for 1 hour in micro-centrifuge tubes with gentle shaking. Non-adherent bacteria were removed by washing with 9 volumes of 15% sucrose-PBS solution at 1000 × g for 5 minutes at 4°C. The adherent bacteria were detected by ELISA or flow cytometry methods as described in section **3.4.1** or **3.4.3,** respectively. The primary antibody used for detection was rabbit anti-*H. pylori* IgG (1:20000 diluted, DAKO) and the secondary antibodies used were goat anti-rabbit IgG, HRP conjugate (1:2000 diluted, DAKO) or goat anti-rabbit, FITC conjugate (1:500 diluted, Sigma).

3.11 Animal study of *Helicobacter pylori*

3.11.1 Inoculation procedure of *H. pylori* in mice

The animal study was approved by the Animal Experimental Ethic Committee, National University of Singapore. Both *H. pylori* SS1 *hsp20* wild type and *H. pylori* SS1 *hsp20::aphA* deficient strains were inoculated into BALB/c mice (20 – 25 g, < 4 weeks of age & single sex type, female). The inoculation procedure was followed as suggested by Smythies *et al*. (2000). After overnight fasting, 1×10^8 CFU *H. pylori* BHI broth culture in 300 μl was administrated into each animal, three successive challenges were carried out on alternate days. A total of 15 mice were inoculated for each *H. pylori* strain and 9 mice fed with BHI broth alone serving as negative controls (Table 3.5). At 2, 4 and 8 weeks after inoculation, mice were sacrificed, stomachs were removed and dissected longitudinally into three equal parts for the detection of *H. pylori* by microbiological, histopathological and RT-PCR assays. Whole blood from the sacrificed mice was drawn and antibody responses to *H. pylori* were analyzed.

Table 3.5 Procedures for challenging mice with *H. pylori*

	wild type *H. pylori* SS1	***hsp20*-isogenic *H. pylori* SS1**	**Negative control**
Number of bacteria inoculated (CFU)	1×10^8 to each animal per dose; 3 doses on alternate day	1×10^8 to each animal per dose; 3 doses on alternate day	BHI broth
No of animals inoculated	15	15	9
Sacrificing schedule (weeks)	2, 4 & 8	2, 4 & 8	2, 4 & 8

3.11.2 Analysis of mouse gastric biopsy

3.11.2.1 Microbiological analysis

One third of the stomach mucosa (washed with PBS buffer four times) was minced using glass slide in 50 µl BHI broth, the minimum suspension was swabbed onto CBA plates supplemented with 4 antibiotics: vancomycin 3 µg/ml, trimethoprim 5 µg/ml, nalidixic acid 10µg/ml and amphotericin 2µg/ml (Sigma) as described in the section **3.1.1**. The plates were incubated at 37°C with 5% CO_2 for up to 7 days. After swabbing, the stomach piece was directly transferred into the microfuge tube containing urease test reagent for detecting the presence of *H. pylori* urease.

Following incubation, the plates were examined for the bacterial growth; the suspected *H. pylori* colonies were examined by gram staining, urease, oxidase and catalase tests. The stomach was considered as positive for *H. pylori* when the following three criteria were met: (1) growth of pinpoint, transparent and non-hemolytic colonies on the chocolate blood agar plates; (2) presence of gram negative spiral under the microscope; (3) pin point colonies showed the presence of urease, oxidase and catalase.

The standard protocols were followed for gram staining (Gerald, 1994), urease, oxidase and catalase tests (Clayton & Mobley, 1997). For the urease test, the reagent used comprised 2% (W/V) urea; 1.5 mM $NaH_2PO_4 \bullet H_2O$; 4 mM Na_2HPO_4; 0.075% (V/V) phenol red, the pH of the solution was adjusted to 6.8. When the color of reagent was changed from yellow to pink in the presence of the mice stomach, it was considered as urease positive. For the catalase test, the reagent used was 3% hydrogen peroxide. It was considered as catalase positive when effervescence was observed in the reagent in the presence of bacteria. For the oxidase test, 1% tetro-methyl-p-phenylenediamine-

dihydrochloride aqueous solution was used as substrate. When bacteria culture was placed onto the filter paper soaked with oxidase reagent, the presence of a deep blue color indicated oxidase positive.

3.11.2.2 Histological analysis

The paraffin-embedded stomach biopsy blocks were prepared at the Department of Pathology, National University Hospital, Singapore. The presence of *H. pylori* in the sections of mice gastric tissue was detected using immunohistochemistry technique as described by Jonkers *et al*. (1997). Rabbit anti-*H. pylori* IgG (1:50 diluted, DAKO) and goat anti-rabbit IgG HRP conjugate (1:100 diluted, DAKO) were used as the primary and secondary antibody, respectively in the immuno-staining. 3,3-diaminobenzidine tetrahydrochloride (DAB) was used as the substrate in the enzymatic reaction. The presence of brown color, spiral, rod-shaped bacteria on the luminal surface were considered as histological positive for *H. pylori*.

3.11.2.3 Total RNA extraction and RT-PCR analysis

Total RNA was extracted from a piece of mouse stomach by RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. RNA (~1μg) was digested with RNase free DNase. Reverse transcribed into the first strand of cDNA was carried out at 42°C for 1 hour using random hexamer as primer and AMV Reverse Trancriptase (Promega). The transcribed cDNA was used as the template to amplify *H. pylori* 16S rRNA and *ureC* gene fragments by PCR while the amplification of GAPDH gene fragment was used as the internal control. The primers for the amplification of specific

gene fragments were listed in Table 3.6. The PCR amplification was performed by denaturation at 94°C for 5 minutes followed by 94°C, 30 seconds, 55°C or 50°C or 52°C, 30 seconds, 72°C, 30 seconds for 35 cycles with additional extension at 72°C for 5 minutes. The PCR cycles were carried out on GeneAmp PCR system 2400.

Table 3.6 Primers used in the RT-PCR analysis

Name	Sequences (5' – 3')	Length of DNA fragment (bp)	Tm (°C)	References
16s rRNA	Forward: GGAGGATGAAGGTTTTAGGATTG Reverse: TCGTTTAGGGCGTGGACT	390	55	Rokbi *et al*., 2001
ure C	Forward: AAGCTTTTAGGGGTGTTAGGGGTTT Reverse: AAGCTTACTTTCTAACACTAACGC	294	50	Labigne *et al*., 1991
GAPDH	Forward: ACCACAGTCCATGCCATCAC Reverse: TCCACCACCCTGTTGCTGTA	451	52	Ye *et al*., 1997

3.11.3 Detection of antibody against *H. pylori*

BALB/c mice inoculated with *H. pylori* were sedated and the whole blood was drawn from the sedated mice by cardiac puncture followed the guidelines provided by Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC), U.S.A. Serum was extracted and stored at - 20°C until use. Acid glycine extracted *H. pylori* SS1 protein (prepared according to section **3.5.2.**) was used as antigen to detect the antibody level against *H. pylori* in inoculated mice using ELISA as described in section **3.4.1**. An aliquot of 0.5 μg acid glycine extract (AGE) antigen in 100

μl coating buffer was added into each well of microtiter plate. Different dilutions of mice primary antibody (1:100; 1:200; 1:500 & 1:1000) were added in triplicates. Goat anti-mouse immuno-globulin HRP conjugate (1:2000 diluted, DAKO) was used as the secondary antibody.

3.12 Protein profile of *Helicobacter pylori*

To examine the protein profiles of wild type and *hsp20*-isogenic mutant *H. pylori*, the total protein exact of both *H. pylori* cells were analyzed on SDS-PAGE and 2D gel electrophoresis. The total protein extract of *H. pylori* was extracted according to the protocol as described in section **3.5.1.**

For SDS-PAGE, a total of 15 μg protein extract from each *H. pylori* strain was loaded on 12% gel. The gel with separated protein bands was then stained by silver nitrate. The running of SDS-PAGE and staining of gel followed the protocols as described in section **3.3.2**.

For 2D gel electrophoresis, a total of 150 μg protein extract from each *H. pylori* strain was applied. The IPG strip of broad pH range from 3 to 10 was used in the analysis. The procedure for 2D gel electrophoresis was followed as described in section **3.3.3**. The 2D gel of both wild type and the isogenic mutant *H. pylori* was transblot onto PVDF membrane for Western blotting analysis. The protocol for Western blotting was followed as described in section **3.4.2**. The primary antibody used was anti-rHSP20 antibody (1:800 diluted) while secondary antibody used was goat-anti rabbit IgG (DAKO, 1:2000).

3.13 Status of genes encoding for *Helicobacter pylori* adhesins

3.13.1 DNA sequencing of dinucleotide repeats

To examine the gene status (in-frame or out-of-frame) of three major adhesins [*OipA* (outer inflammatory protein, HP0638), *HopZ* (homologue of porin, HP0009), *SabA* (sialic acid-binding adhesin, HP0725)], the CT dinucleotide repeat regions in the signal sequence of these genes were amplified by PCR. Genomic DNAs of both wild type and *hsp20*-isogenic *H. pylori* strains were used as templates. The conditions for PCR amplification was followed as described by de Jonge *et al*. (2004). The PCR amplified fragments were sequenced using the same method as described in section **3.2.4**. The primers used for PCR amplification and DNA sequencing were listed in Table 3.7.

Table 3.7 Primers used for detecting the functional status of *H. pylori* adhesins

Names	Sequences (5' – 3')	Genes	References
oipA-Fs	CAA GCG CTT AAC AGA TAG GC	HP0638	de Jonge *et al*., 2004
oipA-Rs	AAG GCG TTT TCT GCT GAA GC	HP0638	de Jonge *et al*., 2004
hopZ-Fs	GCC TGA TAT GGG TGG CAT GGG	HP0009	de Jonge *et al*., 2004
hopZ-Rs	ATT TGA TAG CCC GCG CTG AT	HP0009	de Jonge *et al*., 2004
sabA-Fs	TTT TTG TCA GCT ACG CGT TC	HP0725	Lehours *et al*., 2004
sabA-Rs	ACC GAA GTG ATA ACG GCT TG	HP0725	Lehours *et al*., 2004

3.13.2 RT-PCR analysis

To examine the transcription of these adhesins in *H. pylori* cells, total RNAs were extracted from both wild type and *hsp20*-isogenic *H. pylori* strains using RNeasy Mini kit (Qiagen). The reverse transcription of RNA was carried out as described in section

3.11.2.3. The transcribed cDNA was used as template to amplify the presence of adhesins genes by PCR; the amplification of 16s rRNA gene fragment of *H. pylori* was used as the internal control. The PCR amplifications for *oipA*, *hopZ* and *sabA* gene fragments were conducted by denaturation at 94°C for 5 minutes followed by 94°C, 30 seconds; 50°C or 52°C, 30 seconds and 72°C, 30 seconds for 35 cycles with additional extension at 72°C for 5 minutes. The primers used in RT-PCR analysis were listed in Table 3.8.

The presence of *babA2* gene was also examined using PCR. The PCR amplification for *babA2* gene fragment was carried out by denaturation at 94°C for 5 minutes followed by 94°C, 1 minute; 50°C, 1 minute and 72°C, 1 minute for 35 cycles with additional extension at 72°C for 10 minutes. The primers used were in Table 3.8.

Table 3.8 Primers used for RT-PCR of various *H. pylori* adhesins

Names	Sequences (5' – 3')	Length of DNA fragments (bp)	Tm (°C)	References
oipA-F	ATGAGCTCAGCTTTGGGTATAA			
oipA-R	GCGATCAATATCGTATTCATCA	457	50	Tomb *et al*., 1997
hopZ-F	ACTACTACTACTACTAATGACG			
hopZ-R	AATCCTTAAGGCTGCCTCTAAA	611	50	Tomb *et al*., 1997
sabA-F	ATCCACTAATTACCCAACGCAAT			
sabA-R	GTCGTTATAGGCGGTTACGATT	643	52	Tomb *et al*., 1997
**** babA2-F***	AATCCAAAAAGGAGAAAAAGTATGAAA			
**** babA2-R***	TGTTAGTGATTTCGGTGTAGGACA	810	50	Gerhard *et al*., 1999

* These are the primers used for regular PCR.

3.14 Identification of protein interacting with HSP20 in *Helicobacter pylori*

3.14.1 Co-immunoprecipitation (CO-IP) using antibody against rHSP20

The procedure for co-immunoprecipitation analysis was according to that as described by Voland *et al*. (2003). Wild type *H. pylori* and *hsp20*-isogenic *H. pylori* mutant cells were used in this study. The 3-day old *H. pylori* cells were harvested and washed three times with PBS buffer. The cell pellet was suspended in co-immunoprecipetation (CO-IP) buffer and incubated on ice-bath for 1 hour with occasional inversion. The CO-IP buffer comprises 50 mM Tris-HCl pH 7.8, 0.5% (W/V) Triton X-100, 0.5 M NaCl, 10 mM EDTA, freshly prepared 1 mM DTT and protease inhibitor cocktail (Roche). The resultant protein supernatant was subjected to the procedure of co-immunoprecipitation with antibody against rHSP20 (1 μg) and incubated on ice for 2 hours with gentle inversion occasionally. The antibody complex was then pulled down by Protein A sepharose (50 μl slurry) on ice for an additional hour. Protein A beads with antibody complex were settled down by centrifugation briefly and washed three times with CO-IP buffer at 4°C. Then the beads were re-suspended in 50 μl of 2 × SDS sample buffer and subjected to SDS-PAGE analysis as described in section **3.3.2**. The isolated protein band on SDS-PAGE gel was excised and sent for identification using MALDI-TOF mass spectrometry as described in section **3.3.4**. Total proteins of *E. coli* Top10 cells and rabbit pre-immune serum were used as the negative control and internal control, respectively. Four independent experiments were repeated.

3.14.2 Western blotting analysis of CO-IP using different antibodies

According to the results obtained from protein identification, mouse antiserum against rCagA (rCagA: his-tag fused CagA protein fragment corresponding to 1st – 285th amino acids of *H. pylori* 26695 as prepared for this study) and antibody against rHSP20 were used for the Western blotting analysis of CO-IP. Wild type *H. pylori*, *hsp20*-isogenic *H. pylori* mutant and CagA negative *H. pylori* strain 1024 (clinical isolate, served as negative control) were used for this test.

The same procedure for CO-IP was performed as described in section **3.14.1**. The CO-IP protein-antibody mixture was subjected to SDS-PAGE and blotted onto the PVDF membrane. Western blotting was then carried out according to the procedure as described in section **3.4.2**. In the Western blotting analysis of CO-IP, mouse antiserum against rCagA (1:800 diluted) and antibody against rHSP20 (0.5 μg/ml) were used as the primary antibody for probing respectively. Goat anti-mouse Ig HRP conjugate (1:2000 diluted, DAKO) and goat anti-rabbit IgG HRP conjugate (1:2000 diluted, DAKO) were used as the secondary antibody. DAB was used as the substrate for the enzymatic reactions.

3.14.3 RT-PCR analysis of *cagA* transcription in *H. pylori*

Both wild type and *hsp20*-isogenic *H. pylori* strains were used in this test. In RT-PCR analysis, *H. pylori* cells of different ages (3rd & 4th days) were harvested from plates. Total RNA was extracted from each sample using RNeasy Mini kit (Qiagen). The reverse transcription of RNA was carried out as described in section **3.11.2.3**. The transcribed cDNA was used as template to amplify the presence of *H. pylori cagA* gene by PCR; the amplification of 16s rRNA gene fragment and *hsp20* gene fragment were used as the

internal control. The primers for the amplification of *cagA* gene fragment were based on the known sequences of *H. pylori* 26695 (Tomb *et al*., 1997). The forward primer used was: 5'>GGAACGCCATATGATGACTAACGAAACCATTG<3'; the reverse primer used was: 5'>CGCGGATCCTTAATCAATGTCAGCGACTCCC<3'. The PCR amplification conditions for *cagA* gene fragment was carried out by denaturation at 94°C for 5 minutes followed by 94°C, 30 seconds, 52°C, 30 seconds, 72°C, 45 seconds for 35 cycles with additional extension at 72°C for 10 minutes. The target *cagA* gene fragment was 852 bp. The primers for amplification of 16s rRNA was the same as in Table 3.7 and PCR amplification was performed as described in section **3.11.2.3**. The primers and PCR amplification for *hsp20* gene fragment was followed as described in section **3.2.4.1**.

3.14.4 Detection of CagA in different *H. pylori* sub-cellular fractions

Wild type *H. pylori*, *hsp20*-isogenic *H. pylori* and CagA negative *H. pylori* 1024 (served as negative control) were used in this test. To analyze the presence of CagA protein in *H. pylori*, different sub-cellular fractions of *H. pylori* [total protein (TP), acid glycine extract (AGE) and outer membrane fraction (OMP)] were extracted according to the methods as described in section **3.5**. The same amount of protein (10μg) from each fraction was subject to SDS-PAGE and Western blotting as described in section **3.4.2**. The primary antibody used was antiserum against rCagA while rabbit antiserum against HSP60 was used as the internal control. The secondary antibody used was goat anti-mouse immunoglubilin HRP conjugate and goat anti rabbit IgG HRP conjugate (DAKO, 1: 2000 diluted). DAB was used as the substrate for the enzymatic reactions.

To examine whether the presence of HSP20 affects the presence of CagA protein in different *H. pylori* sub-cellular fractions, a test was further carried out with the addition of rHSP20 in *H. pylori* cultures. The same experiment was carried out for the *H. pylori* cultured with the addition of different concentrations of rHSP20 (0.1 μg/ml, 0.5 μg/ml and 1 μg/ml) in the culture media. All cultures were incubated for further 24 hours after the addition of rHSP20.

3.14.5 Detection of antibody against CagA in *H. pylori* inoculated mice

ELISA methods were employed in this study as described in section **3.4.1**. Recombinant CagA (rCagA: His-tag fused CagA protein fragment corresponding 1st – 285th amino acids of *H. pylori* 26695) prepared earlier in our lab was used as the antigen to detect the antibody against CagA protein in *H. pylori* infected mice in section **3.11**. An aliquot of 0.5 μg (rCagA) in 100 μl coating buffer was added into each well of micro-titer plates. Different dilutions of mice primary antibody (1:100; 1:200; 1:500 & 1:1000) were added in triplicates. Goat anti-mouse Ig HRP conjugate (1:2000 diluted, DAKO) was used as the secondary antibody for detection. The detection of antibody against Le (X) and Le (Y) in *H. pylori* infected mice was served as the internal control. The antigens used were synthetic Le (X) and (Y) (IsoSep, Sweden) at 0.1 μg/well. The procedure for the detection was carried out according to the protocols as described by Zheng *et al*. (2000).

3.15 DNA sequencing of *hsp20* gene

A total of 225 *H. pylori* isolates obtained from different geographical regions and ethnic origins were included in this testing (Table 3.9). Among these, 103 strains were isolated from Singapore and 122 strains were from 9 different countries. All *H. pylori* isolates were cultured on CBA plates as described in section **3.1.1**.

A pair of primers corresponding to 50 bp upstream and downstream of *hsp20* (HP0515) of *H. pylori* 26695 genomic sequences was used (Tomb *et al*., 1997). The forward primer was: 5'> CGGAATTCAGATTGAAGTCAAGC <3' while the reverse primer was: 5'> CGGGATCCTGCCCAATGATGTATT <3'. The genomic DNAs were extracted according to the protocol as described in section **3.2.1** and were used as the templates for PCR amplification. The PCR amplification was carried out by an initial denaturation at 94°C for 5 minutes followed by 94°C, 30 seconds, 50°C, 30 seconds, 72°C, 30 seconds for 30 cycles with additional extension at 72°C for 5 minutes. The PCR amplified 643 bp gene fragments were purified by PCR product purification kit and sequenced using BigDye ^{TM} Terminator Cycle Sequencing Ready Reaction Kit and sequenced using ABI 100 model 377 DNA sequencer.

Table 3.9 Geographical distribution and clinical status of 225 strains used for *hsp20* gene sequencing

Regions	Countries	Clinical diagnosis	No of strains
Asian	**Singapore** (n=103)	NUD	28
		PUD	75
	Japan (n=43)	NUD	20
		PUD	23
	India (n=6)	NUD	2
		PUD	4
	Hong Kong (n=6)	N.K.	6
Non-Asian	**Sweden** (n=16)	NUD	6
		PUD	10
	Peru (n=12)	NUD	12
	Spain (n=14)	N.K	14
	Lithuania (n=12)	NUD	6
		PUD	6
	Costa Rica (n=9)	N.K.	9
	Australia (n=4)	NUD	1
		N.K.	3

NUD: non-ulcer dyspepsia; PUD: peptic ulcer; NK: not known.

3.16 Phylogenetic analysis

The DNA sequences of *hsp20* from 225 *H. pylori* isolates obtained and the two known genomic DNA sequences strains (*H. pylori* 26695, genebank accession no: AE000566, Tomb *et al*., 1997; *H. pylori* J99, Genebank accession no: AE001480, Alm *et al*., 1999) were aligned and the corresponding amino acid sequences were deduced by using program Bioedit (Hall, 1999). Multiple alignments of sequences were conducted by ClustalX (ver 1.81) (Thompson *et al*., 1997). The GC content, polymorphic sites, percentages of the mean differences between pairs of strains at synonymous nucleotide

positions (K_S) and nonsynonymous nucleotide positions (K_a) were calculated using DNASP version 3.5 (Rozas & Rozas, 1999).

PHYLIP (the PHYLogeny Inference Package) Version 3.6 was used to generate phylogram (Felsenstein, 1989) based on the *hsp20* gene of 227 *H. pylori* strains. The maximum likelihood (ML) algorithm was chosen and performed following the procedures proposed by Baxevanis & Oullette (2001). A bootstrap analysis (100 replicates) was performed to evaluate the topology of the phylogenetic trees. The nucleotide divergence between groups was estimated by using Jukes-Cantor methods in DNASP 3.5.

3.17 HSP20 protein structure predicted by homology modeling

Homology modeling (also called comparative protein modeling) was chosen to predict the structure of HSP20 protein. It is a process by which the building of a 3-D model of a target sequence is based on a homologue (with at least 30% identity) which structure has been experimentally solved (either by X-ray crystallography or solution nuclear magnetic resonance, NMR). Homology modeling is a rapid way to identify the ("probable homologous") structure of a protein and its possible function based on homologous protein (Baker & Sali, 2001). In the process of homology modeling, the sequence of target protein with unknown 3-D structure is used as a query to search the defined database (e.g. Protein Data Bank, PDB); when the appropriate template with resolved structure is matched, an alignment between the sequences of target and template will be created and the possible structure will be predicted based on the alignments. This

process can be completed with a special program (e.g. Protein Explorer) in which a specific database is defined and incorporated with tools of visualization for 3-D structure.

In this study, SWISS-MODEL Server (an automated comparative protein modeling server) (Guex & Peitsch, 1997) available at http://www.expasy.org/swissmod/ was used to predict protein structure of HSP20. The amino acid sequence of HSP20 (HP0515 of *H. pylori* 26695, Tomb *et al*., 1997) was used as a query for matching homologous templates from ExPDB database. The two best-scored templates chosen for modeling are ATP-dependent protease HslV of *Haemophilus influenzae* showing 57% identity (PDB accession code: 1kyiL, Sousa *et al*., 2002) and HslV of *Escherichia coli* showing 49% identity (PDB accession code: 1e94A, Song *et al*., 2000). The predicted protein structure was evaluated by WHATCHECK program (Rodriguez *et al*., 1998) that suggested the stereochemistry and energetic parameters of the model was acceptable.

3.18 Structure comparison of substitutions at 14^{th} – 16^{th} amino acid residues of HSP20

To examine whether the substitutions at 14^{th} – 16^{th} amino acid residues affect the protein structure of HSP20, the amino acids sequences of *hsp20* that represent seven major types of substitutions respectively were used for homology modeling using SWISS-MODEL as described in section **3.16**. Various types of amino acid substitutions were examined based on its position in the 3-D structure and secondary structure predicted.

4. RESULTS

4.1 Preparation of recombinant HSP20 (rHSP20)

4.1.1 Construction of rHSP20 expression vector

hsp20 was cloned into pET 16b expression vector as shown in Figure 4.1A. The 543 bp *hsp20* gene fragment of *H. pylori* NCTC 11637 as amplified by PCR as shown in Figure 4.1B. The target gene fragment was inserted into pET 16b expression vector at BamHI restriction enzyme site that was fused at the downstream of Histidine-tag. The total length of recombinant plasmid is 6255 bp as shown in Figure 4.1C. The clone with the correct gene orientation was shown to have a 533bp fragment (Fig 4.1C, lane 3). Complete DNA sequences of *hsp20* and its deduced amino acids sequences are illustrated in Figure 4.2.

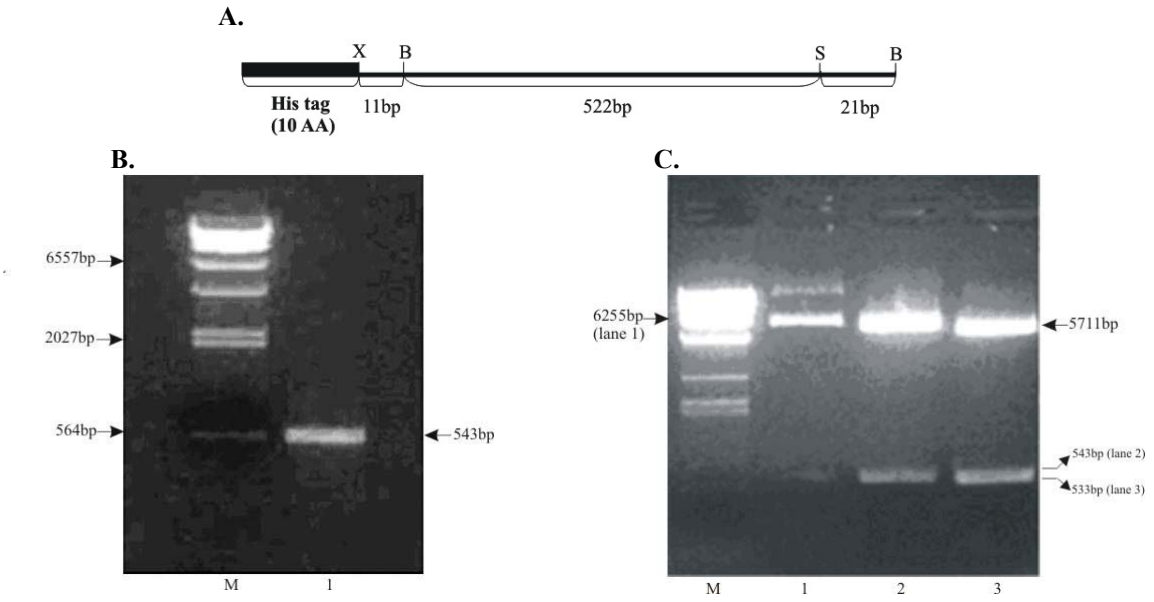


Figure 4.1 Construction and identification of *hsp20* expression vector

A, Diagrammatic representation of pET-16b-*hsp20* plasmid; X, XhoI; B, BamHI; S, SspI; **B**, PCR amplified *hsp20* gene fragment; lane 1, target gene fragment (543bp). **C**, Dissection of recombinant pET16b-*hsp20* plasmid identification; lane1, uncut recombinant palsmid (pET16b + *hsp*20, 6255bp; lane 2, recombinant plasmid digested with BamHI; lane 3, recombinant plasmid digested with XhoI + SspI giving a fragment of 533bp; lane M, λ Hind III DNA marker.

```
    Met Phe Glu Ala Thr Thr Ile Leu Gly Tyr Arg Gly Glu
  1 atg ttt gaa gcg acg acg att cta ggc tat aga ggg gaa

    Phe Asp Asn Lys Lys Phe Ala Leu Ile Gly Gly Asp Gly
 40 ttt gat aat aaa aag ttc gcg ctc att gga ggc gat ggg

    Gln Val Thr Leu Gly Asn Cys Val Val Lys Ala Asn Ala
 79 cag gta act ttg ggt aat tgc gta gtc aaa gcc aat gcg

    Thr Lys Ile Arg Ser Leu Tyr His Asn Gln Val Leu Ser
118 aca aaa atc agg agc ttg tat cac aac cag gtt tta agc

    Gly Phe Ala Gly Ser Thr Ala Asp Ala Phe Ser Leu Phe
157 ggg ttt gcc gga agc acc gca gac gct ttt agt ttg ttt

    Asp Met Phe Glu Arg Ile Leu Glu Gly Lys Lys Gly Asp
196 gat atg ttt gaa cgc att tta gag ggc aaa aag gga gat

    Leu Phe Lys Ser Val Val Asp Phe Ser Lys Glu Trp Arg
235 ttg ttt aaa agc gtg gtg gat ttc agc aaa gaa tgg cgc

    Lys Asp Lys Tyr Leu Arg Arg Leu Glu Ala Met Met Ile
274 aaa gat aag tat tta cgc cga cta gaa gcg atg atg atc

    Val Leu Asn Leu Asp His Ile Phe Ile Leu Ser Gly Thr
313 gtt tta aat tta gat cac att ttc att ttg agc ggc acg

    Gly Asp Val Leu Glu Ala Glu Asp Asn Lys Ile Ala Ala
352 ggc gat gtt tta gag gct gaa gac aat aag atc gct gct

    Ile Gly Ser Gly Gly Asn Tyr Ala Leu Ser Ala Ala Arg
391 att ggg agt ggg ggg aat tac gcc tta agc gcg gct agg

    Ala Leu Asp His Phe Ala His Leu Glu Pro Arg Lys Leu
430 gct tta gat cat ttc gct cat tta gag cct aga aaa ctt

    Val Glu Glu Ser Leu Lys Ile Ala Gly Asp Leu Cys Ile
469 gta gaa gag tcc tta aaa atc gca ggg gat ctt tgc att

    Tyr Thr Asn Thr Asn Ile Lys Ile Leu Glu Leu End
508 tac acc aac acg aat att aaa att ttg gag ctt taa
```

Figure 4.2 DNA sequence of *hsp20* and deduced amino acid sequence of *Helicobacter pylori* NCTC11637 HSP20

The full length of *hsp20* is 543 bp encoding 180 amino acids.

4.1.2 Expression and purification rHSP20 protein

The maximum expression level of recombinant HSP20 (rHSP20) was achieved at 3 hours after induction with 0.4 mM IPTG in *E. coli* BL-21 cells (Figure 4.3A). The expressed rHSP20 was about 10% – 15% of total proteins after induction. The estimated molecular weight of expressed rHSP20 protein is 23 kDa (Figure 4.3A). The expressed rHSP20 protein existed mainly in inclusion body and dissolved in 6 M urea-PBS buffer (Figure 4.3B). Recombinant HSP20 was eluted from His-tag affinity chromatography column with imidazole at the concentration gradient range of 0.3 – 1 M in the presence of 6 M urea (Figure 4.3B). The estimated percentage of expressed rHSP20 in total cell extracts ranged from 5% -10% based on total protein and recovery efficiency of inclusion body was about 20%. Through MS Q-TOF analysis, the amino acid sequence of recombinant rHSP20 was identified as heat shock protein HslV (HP0515) (Figure 4.4).

4.2 Preparation and characterization of antibody against rHSP20

Antibody against rHSP20 raised in two immunized rabbits was detected using ELISA as shown in Figure 4.5. It is shown that the antibody titer was increased at 4 weeks for the rabbit immunized with 120 μg antigen. In contrast, the antibody titer only increased after 6 weeks for the rabbit immunized with 150 μg. There is a bimolar peak of antibody production at the 72^{nd} and 85^{th} days for the rabbit immunized with 120 μg rHSP20. However, there is only one peak at the 72^{nd} day in rabbit immunized with 150 μg rHSP20. The highest antibody titer detected was over 1:6400 regardless of the dosage of antigen used. However, it was shown that immunizing with 120 μg produced higher antibody titer throughout the course of immunization (Figure 4.5).

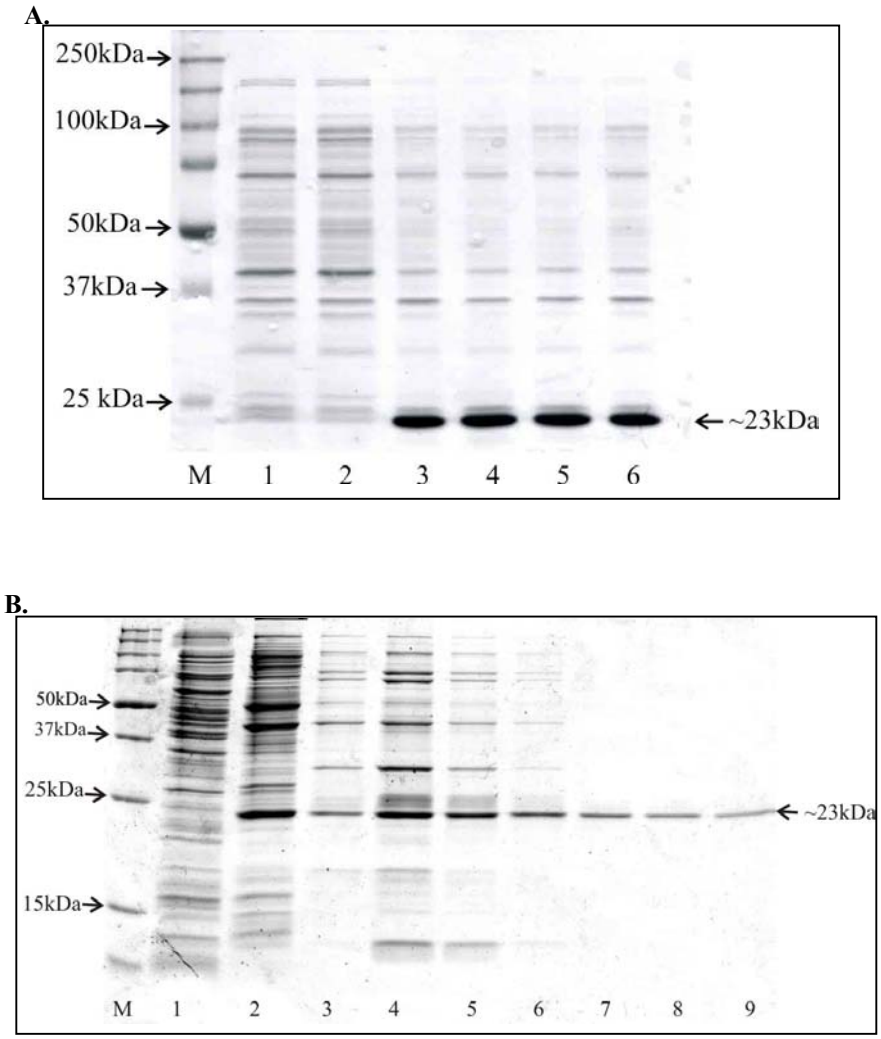


Figure 4.3 Expression and purification of recombinant HSP20 in *E.coli* BL-21

A, Expressed rHSP20 by IPTG induction as run on 10% SDS-PAGE; lanes 1 & 2, proteins of un-induced cells; lanes 3 & 4, IPTG induction for 3 hours; lanes 5 & 6, IPTG induction for 4 hours. **B**, Purified rHSP20 as run on 15% SDS-PAGE; lane 1, supernatant of sonicated cells; lane 2, cell pellet solubilized by 6 M Urea; lanes 3 – 9, fractions eluted from His-tag column in ascending gradient with concentration of imidazole ranged from 0.3 – 1M (in the presence of 6M Urea); M, Prestained Precision Protein Standards (Biorad).

A.

MGHHHHHHHHHHSSGHIEGRHMLEDPFEATTILGYRGELNHKKFALIGGDGQVTLGNCVVK
ANATKIRSLYHNQVLSGFAGSTADAFSLFDMFERILESKKGDLFKSVVDFSKEWRKDKYLRR
LEAMMIVLNFDHIFILSGMGDVLEAEDNKIAAIGSGGNYALSAARALDHFAHLEPRKLVEESL
KIAGDLCIYTNTNIKILEL

B.

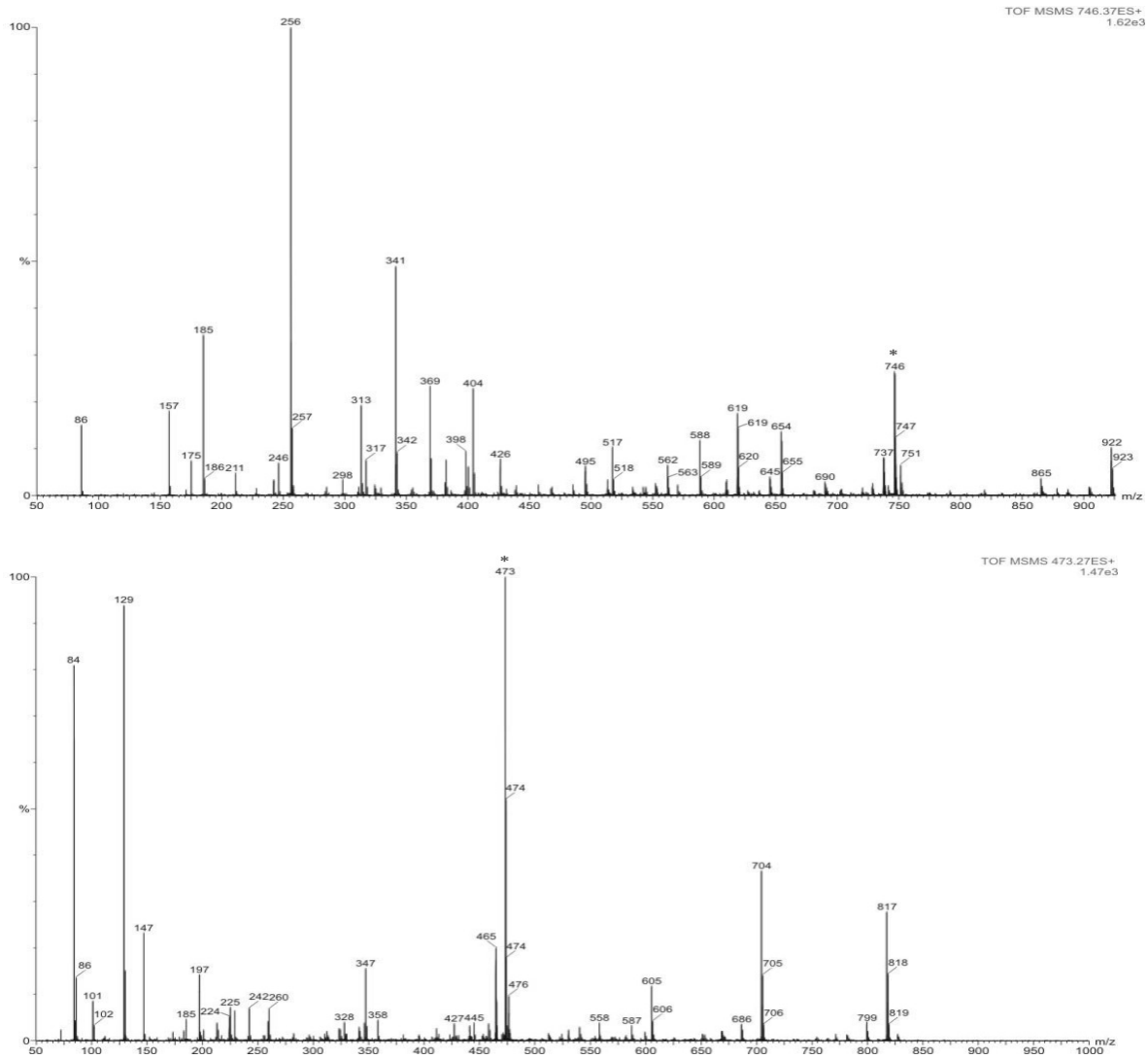


Figure 4.4 Protein identification of rHSP20 by MS Q-TOF analysis

A, The 7 matched peptides (6 – 18 aa) with HP0515 were highlighted in various colors; two representative sets of peptides were identified by mass spectrometry manually. The amino acids in box denote the fused His-tag. **B**, The mass spectrum of the representative peptides (the mass of the peptides were indicated in asterix).

Western blotting (WB) analysis showed different affinity to various *H. pylori* cellular fractions in assaying the binding specificity of antibody to antigens. An intense protein band of ~18 kDa was shown in AGE preparation as compared to proteins extracted by lysis buffer (Figures 4.6A & B). A specific band of the similar molecular weight was observable in the outer membrane protein fraction as shown in Figure 4.6A. Similarly, in the WB of cytoplasmic fraction, a dim band of ~ 18 kDa showed up (Figure 4.6C). In contrast, when HSP60 antiserum was used as the internal control probe, a band of slightly > 50 kDa was detected in both AGE and cytoplasmic fractions (Figure 4.6C).

Interestingly, two-dimensional gel electrophoresis (2-DE) showed the presence of a specific protein spot (Figure 4.7A) with the help of WB using rHSP20 antibody as probe. The specific protein spot at pH of ~5.5 with a molecular weight of ~18 kDa was evident (Figure 4.7B).

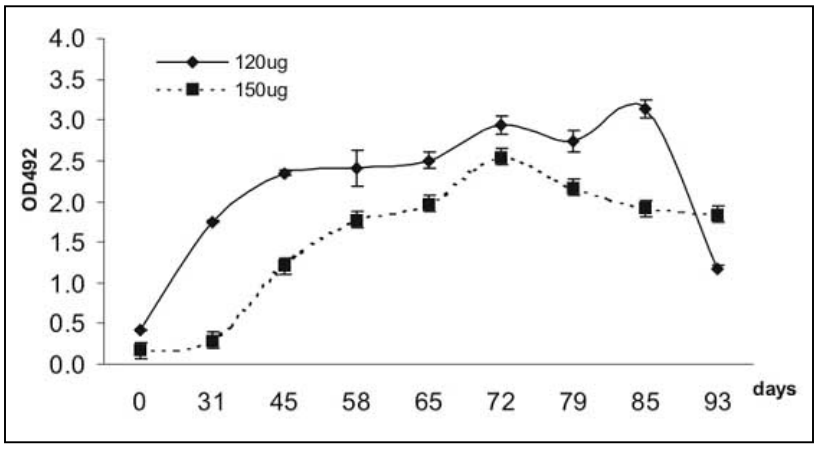
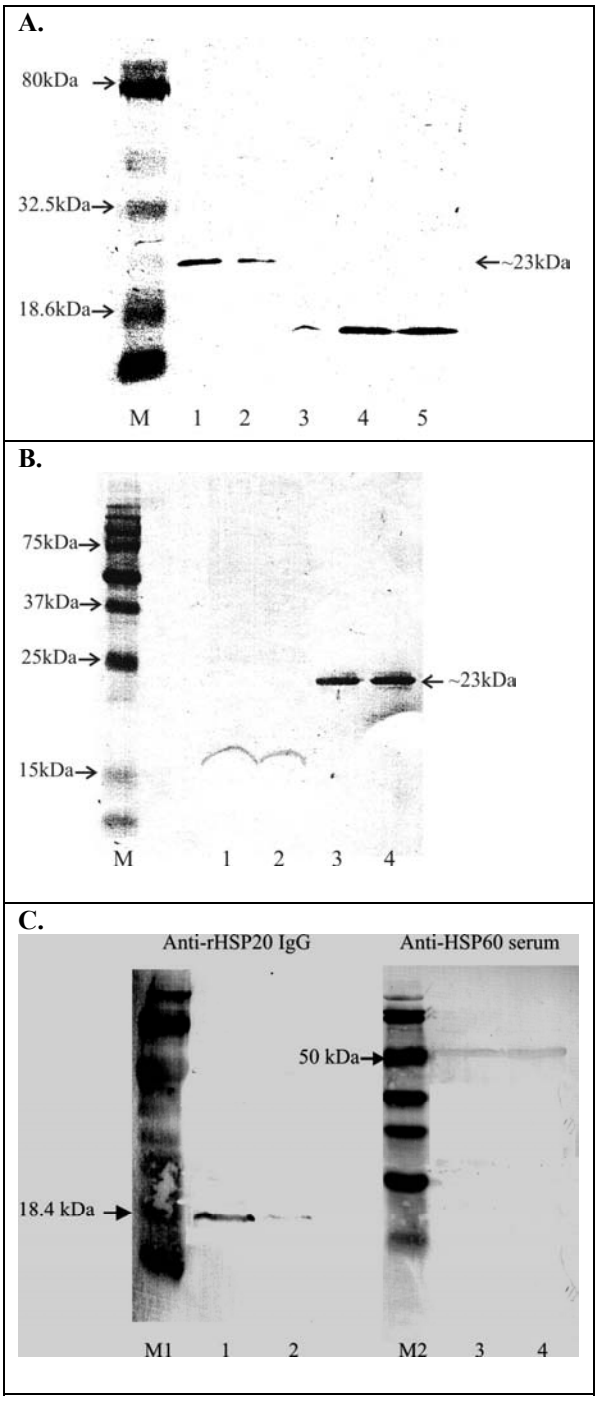


Figure 4.5 Antibody production profile

Antibody titer against rHSP20 detected at different time points. The graph was plotted based on the values of 1:100 diluted serums (showing peak at 72nd day when immunized with 150 µg rHSP20 and at 72nd & 85th day when immunized with 120 µg rHSP20).



Lanes 1 & 2, purified rHSP20 protein (2 µg/ lane was loaded, used as positive control); lane 3, *H. pylori* outer membrane protein fraction (15 µg/lane was loaded); lanes 4 & 5, *H. pylori* acid glycine extract (15 µg/lane was loaded).

Lanes 1 & 2, *H. pylori* cells total proteins extracts (15 µg/lane was loaded); lanes 3 & 4, purified rHSP20 protein (2µg/lane was loaded, positive control).

Lanes 1 & 3, acid glycine extract (AGE) of *H. pylori* (15 µg/lane was loaded); lanes 2 & 4, cytoplasmic fraction (CF) of *H. pylori* (15 µg/lane was loaded); M1, Kaleidoscope Polypeptide Standards (Biorad); M & M2, Prestained Precision Protein Standards (Biorad); lanes 1& 2 were probed with rHSP20 antibody; lanes 3 & 4 were probed with *H. pylori* - HSP60 antiserum.

Figure 4.6 Western blotting of different sub-cellular fractions of *H. pylori* using antibody against rHSP20 as probe

A 15% SDS-PAGE was used for the analysis.

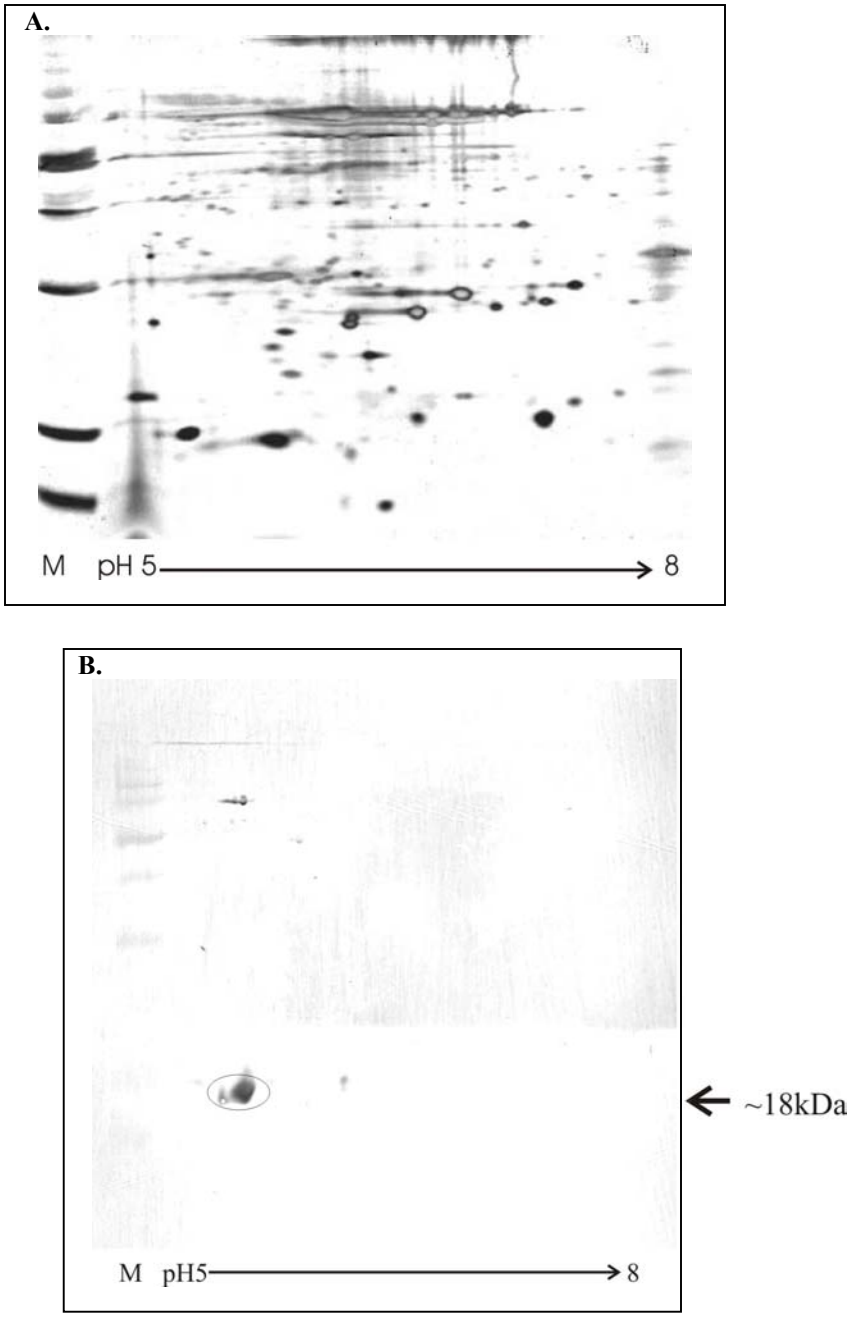


Figure 4.7 Two-dimensional gel electrophoresis and Western blotting of acid glycine extract of *H. pylori*

Based on the ProtParam tool, HSP20 shows preponderance for pI ~6, the linear 7-cm, pH 5-8 IEF strips (Bio-rad) were used ; 15% SDS-PAGE was run for Western blotting analysis and 15 µg protein was applied for each test. **A,** 2-DE of AGE (silver staining); **B,** Western blotting of 2-DE of AGE; M, unstained Precision Protein Standards (Biorad).

4.3 Localization of HSP20 in *Helicobacter pylori*

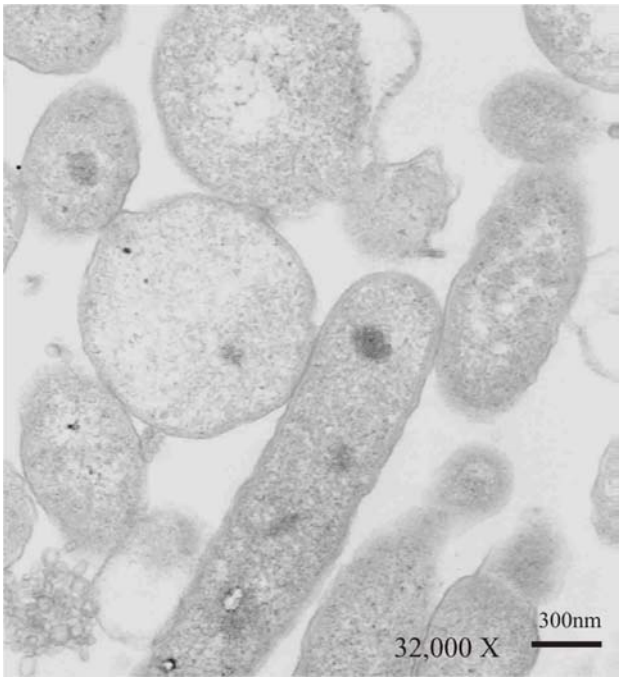
4.3.1 Identified by Western blotting

The results of Western blotting using antibody against rHSP20 to probe different *H. pylori* cellular fractions showed that HSP20 is mainly present in the surface fraction of *H. pylori* as identified in the AGE and OMP fractions (Figures 4.6A). In contrast, using anti-serum against HSP60 as the internal control, HSP60 is present in both AGE and cytoplasmic fractions (Figure 4.6C).

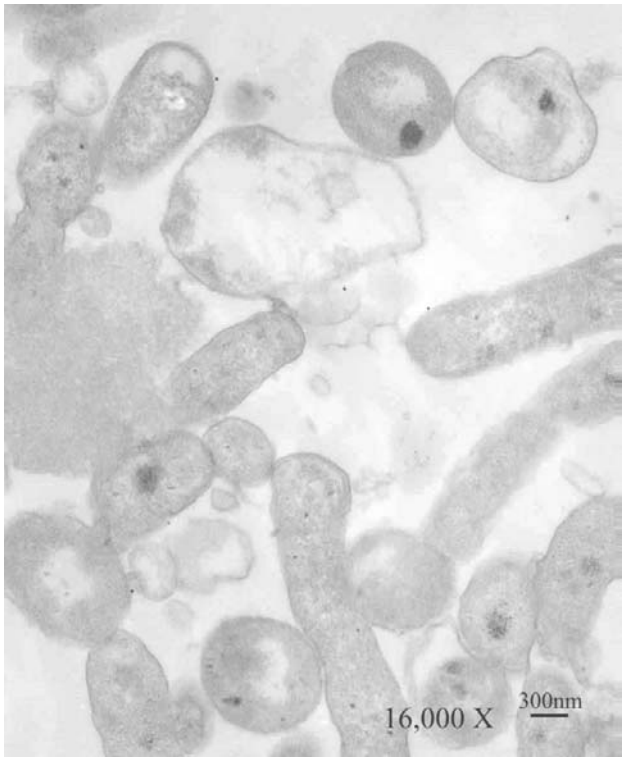
4.3.2 Identified by immuno-gold label TEM

In the immuno-gold labeled TEM study, there was no gold particle observed on the two negative controls, where *H. pylori* was probed with Protein A-gold alone or incubated with pre-immune rabbit serum and probed with Protein A-gold (Figures 4.8A & B). In contrast, when *H. pylori* was incubated with antibody against rHSP20 and Protein A-gold, gold particles of 10nm and 20nm were observed to localize on the cell surface of *H. pylori* (Figures 4.8C & D). Upon Triton X-100 treatment, the cell membrane was partially broken; hence the gold particles were also observed on the remnants of cell membrane (Figure 4.9A & B). However, in Triton X-100 treated *E. coli* cells (serving as the negative control), there was no gold particle observed (Figure 4.9C). But, when Triton X-100 treated *H. pylori* cells were labeled with HSP60 antiserum and 5nm immuno-gold (serving as the internal control), particles were observed mainly in the cytoplasmic regions with some on the surface (Figure 4.9D).

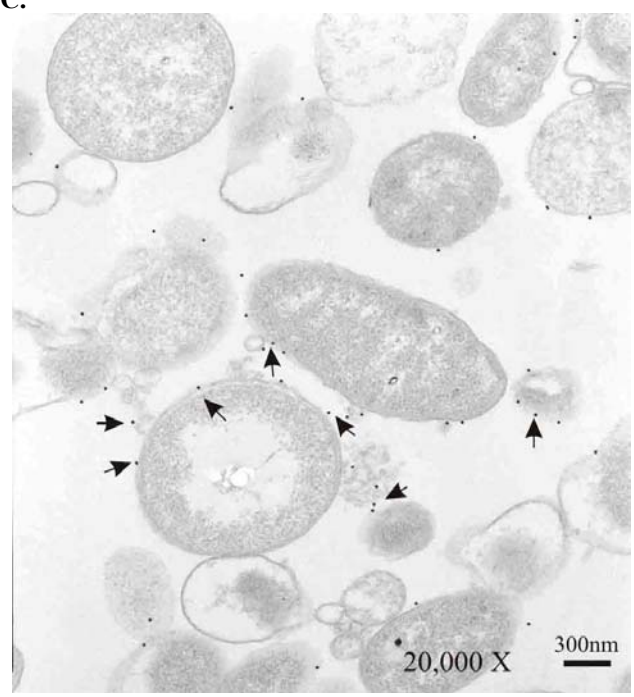
A.



B.



C.



D.

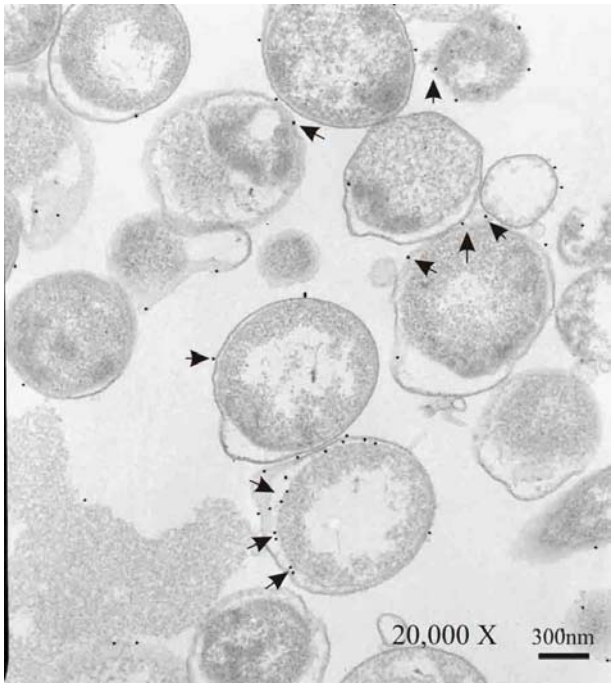
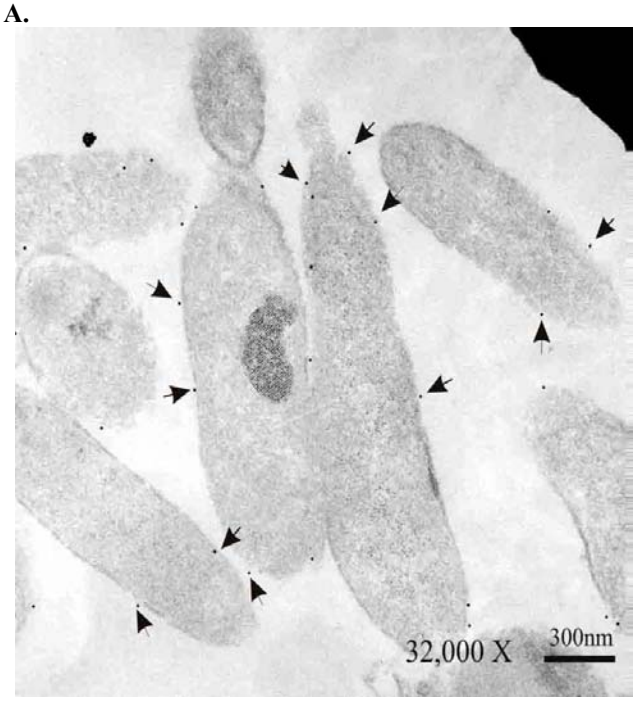


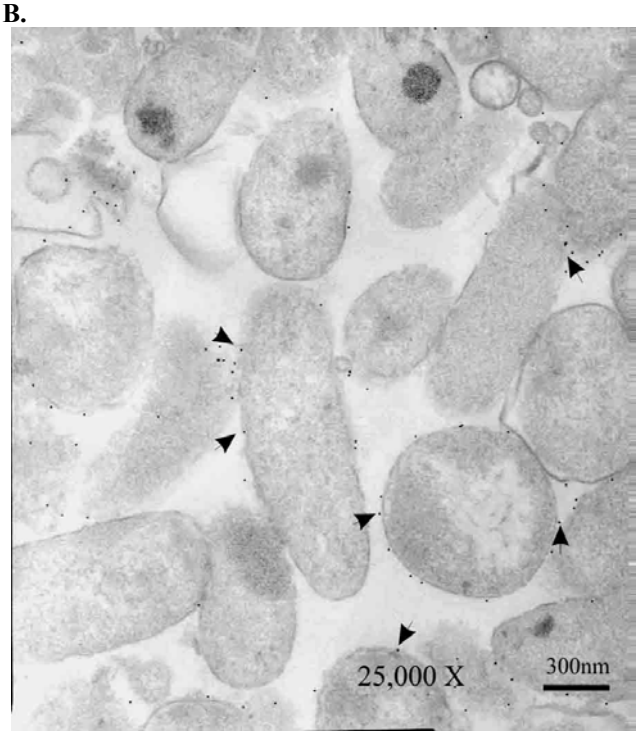
Figure 4.8 TEM of *H. pylori* NCTC 11637 cells labeled with different antibodies

A, labeled with immuno-gold alone (negative control); **B**, labeled with preimmune rabbit IgG and immnuo-gold (negative control); **C,** labeled with antibody against rHSP20 IgG and 10 nm immuno-gold particles; **D,** labeled with antibody against rHSP20 and 20 nm immuno-gold particles. Arrows show sites of localization of HSP20.

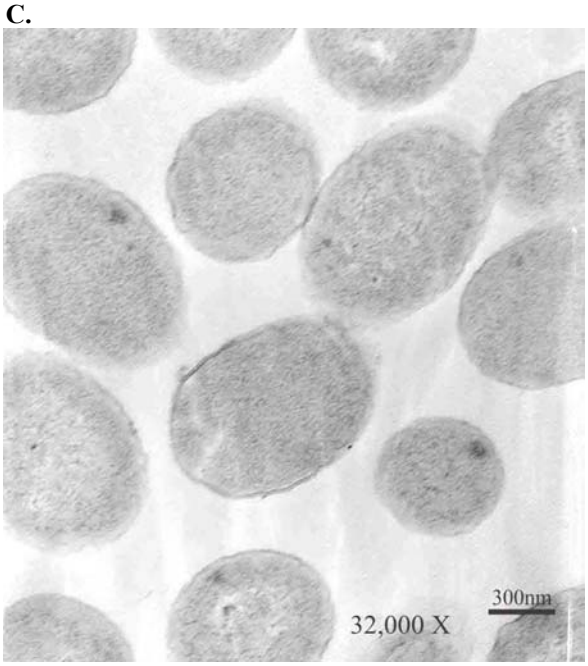
A.



B.



C.



D.

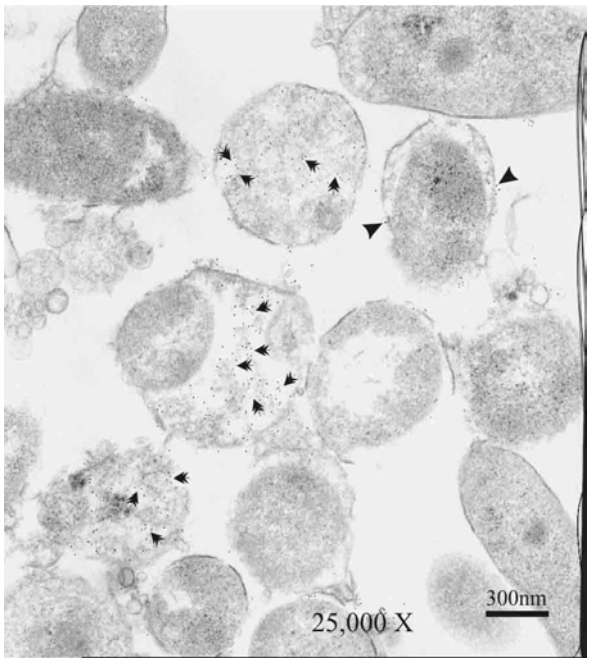


Figure 4.9 TEM of *H. pylori* NCTC 11637 cells labeled with different antibodies after Triton X-100 treatment

A, labeled with rHSP20 antibody and 10 nm immuno-gold after 10 minutes treatment with Triton X-100; **B**, labeled with rHSP20 antibody and 10 nm immuno-gold after 20 minutes treatment with Triton X-100: **C,** *E. coli* cells labeled with rHSP20 antibody after treatment; **D,** labeled with HSP60 antiserum and 5nm immuno-gold after treatment. Single arrows show the surface localization, double arrows show the cytoplasmic localization.

4.4 Antibody titer against HSP20 in patients with gastroduodenal diseases

It is noted that there was no significant difference (p>0.3) examined in the antibody level against rHSP20 between the control subjects and patients with different gastroduodenal diseases. In the disease group, the antibody level against rHSP20 was the same for all patients with or without *H. pylori* infections as well as the normal subjects tested (Table 4.1).

Table 4.1 Sero-prevalence to HSP20 in patients with different gastroduodenal diseases with or without *H. pylori* infection Mean OD_{492}

	Patients with gastroduodenal diseases				Healthy individuals
	PUD		NUD		
***H. pylori* status**[1]	**Positive**	**Negative**	**Positive**	**Negative**	**ND**
OD_{492}	0.211	0.216	0.220	0.231	0.234
(CI)[2]	(0.191-0.232)	(0.202-0.230)	(0.190-0.249)	(0.228-0.233)	(0.195-0.259)
No. of cases	17	8	17	15	32
***p* value**[3]	0.661		0.324		0.574

1, Positive or negative status based on histological examination; 2, Optical density at 492 nm and 95% confident interval of OD value. 3, All p values are from two sided tests. PUD: duodenal ulcer & gastric ulcer; NUD: gastritis & non-ulcer dyspepsia; ND: not done.

4.5 Construction of *hsp20*-isogenic *Helicobacter pylori*

4.5.1 Construction of the gene-targeting vector

Two flanking DNA fragments were amplified by PCR based on *H. pylori* SS1 genomic DNA, 5' fragment is 2700 bp long while the 3' fragment is 2549 bp as shown in

Figures 4.10A & B. Kanamycin resistant gene (*aphA*) was obtained by digestions with EcoRI restriction enzyme on vector pILL 600 (kindly provided by A Labigne, Pasteur Institute, Paris, France) as shown in Figure 4.10C.

These three DNA fragments were separately inserted into cloning vector pBluescript SK(+) by BamHI + PstI, SalI + ApaI and EcoRI sites respectively and identified by resctirction enzyme digestion and sequencing (Figure 4.11). Recombinant pBluescript SK(+) with insertion of *aphA* gene (pBS- *aphA***)** which was 4340 bp in length was chosen as backbone for ligation of the two flanking fragments. The total length of recombinant vector with insertion of *aphA* gene and 5' fragment (pBS-5'-*aphA*) was 7040 bp, which was identified by BamHI + PstI restriction enzyme digestion as shown in Figure 4.12A. The recombinant vector with insertion of *aphA* gene together with both 5' and 3' flanking fragments (pBS-5'-*aphA*-3') was 9589 bp, which was identified by SalI + ApaI restriction enzyme digestion (Figure 4.12B) and sequencing. The sequences of HP0513 from *H. pylori* SS1 shows 90% identity to *H. pylori* 26695 while the rest HP0514 shows 96%; HP0515 shows 97%, HP0516 shows 98% and HP0517 shows 92% identity respectively to the corresponding gene fragments of *H. pylori* 26695.

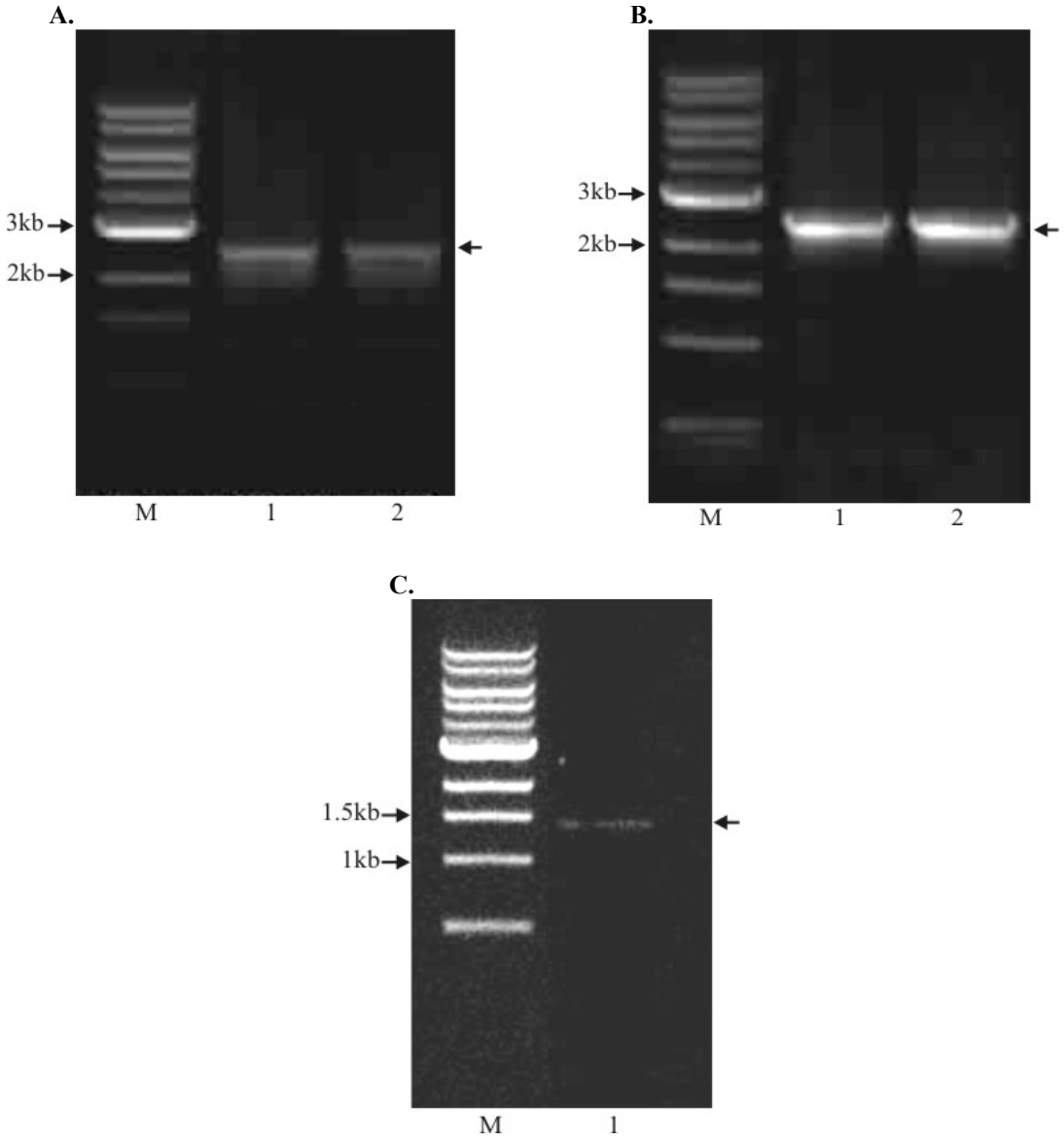


Figure 4.10 Amplification of flanking DNA fragments and extraction of *aphA* gene

A, PCR amplification of 5' flanking fragment of HP0513, 0514 & part of 0515 which was 2700 bp (lanes 1 & 2); **B**, PCR amplification of 3' flanking fragment of part of HP0515, 0516 & 0517 which was 2549 bp (lanes 1 & 2); **C**, Kanamycin resistant gene (*aphA*) fragment as digested by EocRI on pILL600 which was 1340 bp (lane 1); M, 1 kb DNA ladder (NEBiolabs, Beverly, MA, USA).

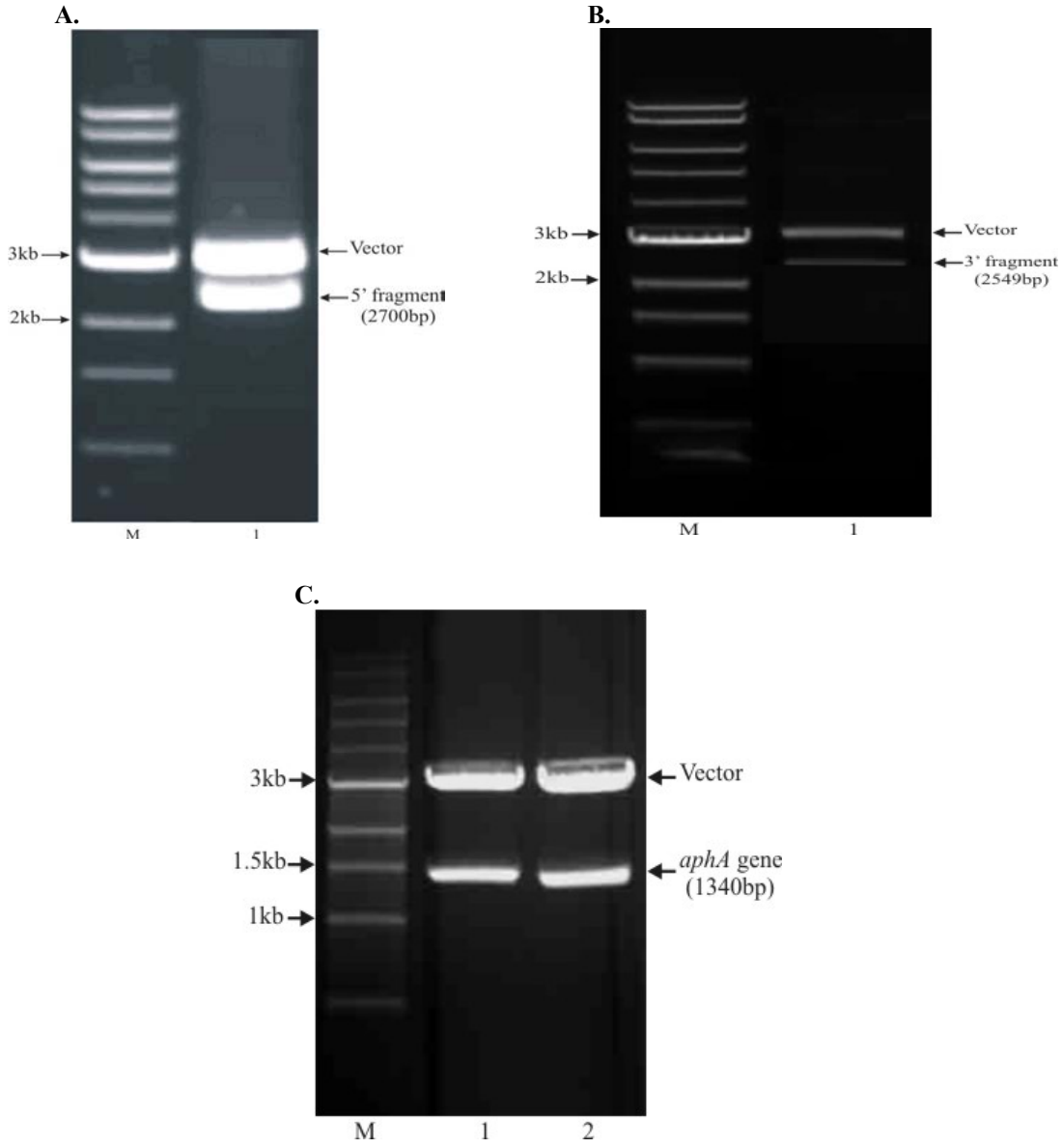
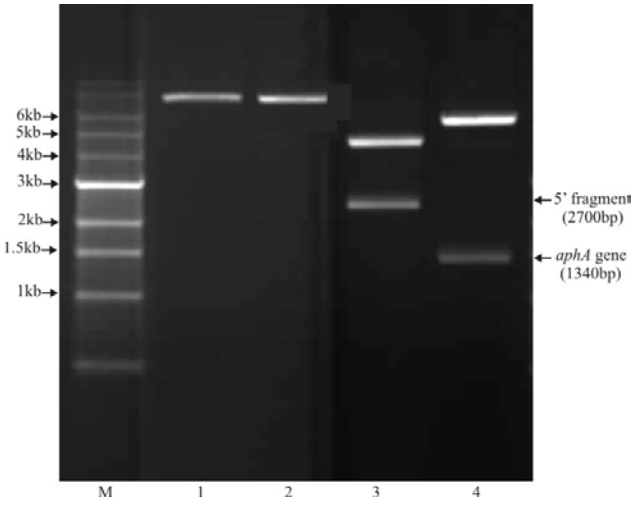


Figure 4.11 Identification of recombinant plasmids

A, Recombinant plasmid with insertion of 5' flanking fragment digested with BamHI + PstI (lane 1); **B**, Recombinant plasmid with insertion of 3' flanking fragment digested with SalI + ApaI (lane 1); **C**, Recombinant plasmid with insertion of *aphA* gene from pILL600 digested with EcoRI (lanes 1 & 2); M, 1 kb DNA ladder.

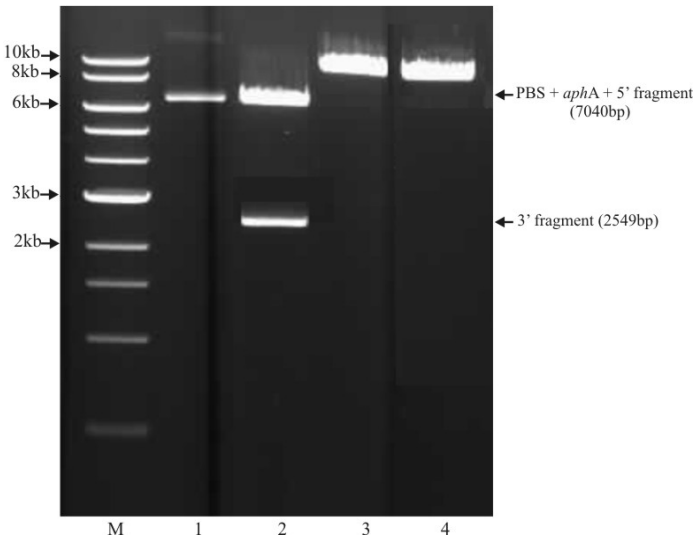
A.



A, Identification of recombinant plasmid with insertion of 5' flanking fragment & *aphA* gene (pBS-5'-*aphA*)

Lanes 1 & 2, pBS-5'-*aphA* digested with BamHI & PstI respectively giving a single 7040 bp fragment; lane 3, pBS-5'-*aphA* digested with BamHI + PstI releasing the 5' fragment of 2700 bp from the vector; lane 4, pBS-5'-*aphA* digested with EcoRI releasing the *aphA* fragment of 1340 bp from the vector; M, 1 kb DNA ladder.

B.



B, Identification of recombinant plasmid with insertion of two flanking fragments and *aphA* gene (pBS-5'-*aphA*-3')

Lane 1, uncut recombinant plasmid pBS-5'-*aphA*-3'; lane 2, pBS-5'-*aphA*-3' digested with SalI + ApaI releasing the 3' fragment of 2549 bp from the vector pBS-5'-*aphA*; lane 3 & 4, pBS-5'-*aphA*-3' digested with SalI & ApaI respectively giving a single 9589bp fragment; M, 1 kb DNA ladder.

Figure 4.12 Identification of recombinant plasmid pBluesript SK with insertion of various gene fragments

4.5.2 Identification of *hsp20*-isogenic *H. pylori*

After the transformation of *H. pylori* SS1 with *hsp20::aphA* gene-targeting vector, 5 kanamycin resistant colonies were selected from the kanamycin containing (25 μg/ml) chocolate blood agar (CBA) plates. The genomic DNA of these 5 clones showed DNA fragments that contain *aphA* gene fragment of different sizes when amplified using different primers (Figure 4.13A-C). When a pair of primers T7 (from vector) and KO3 (from 3' flanking fragment) were used, there was no band amplified from the genomic DNA of the 5 clones as compared with the positive control in which there was a band of 2614 bp amplified based on the targeting vector (Figure 4.13D). This result excluded the possibility of the integration of targeting vector in *H. pylori* genome.

When the genomic DNA of the 5 kanamycin resistant clones were Southern blotted, the genomic DNA fragments with insertion of kanamycin resistant cassette were highlighted by the probe of *aphA* gene (1.34 kb) while the band was absent in the negative control of *H. pylori* SS1 genomic DNA (Figure 4.14A). Similarly, there was no signal detected in the 5 clones when probed with pBluescript SK plasmid DNA (Figure 4.14B). This result further confirmed that the acquisition of kanamycin resistance in the 5 selected *H. pylori* clones is not caused by the integration of targeting vector in bacterial genome but by the insertion of kanamycin resistant gene at *hsp20* gene locus in *H. pylori* genome.

One of the clones was chosen for the expression of HSP20 using Western blotting. By Western blotting, the protein band about ~ 18 kDa was absent from the clone but a dark band of 18 kDa was present in the positive control (*H. pylori* SS1) when probed with antibody against rHSP20 (Figure 4.15A). As the internal control, a specific intensive

band ~ 60 kDa was present in both kanamycin resistant clone and the positive control when probed with anti-HSP60 serum (Figure 4.15B).

The results of PCR amplification, Southern blotting and Western blotting show that homologous recombination occurred between the targeting vector and the bacterial genome at the gene locus of *hsp20*. The transcription of *hsp20* gene was disrupted by kanamycin resistant gene (*aphA*). The obtained 5 kanamycin resistant clones were shown to be *hsp20*-isogenic *H. pylori*.

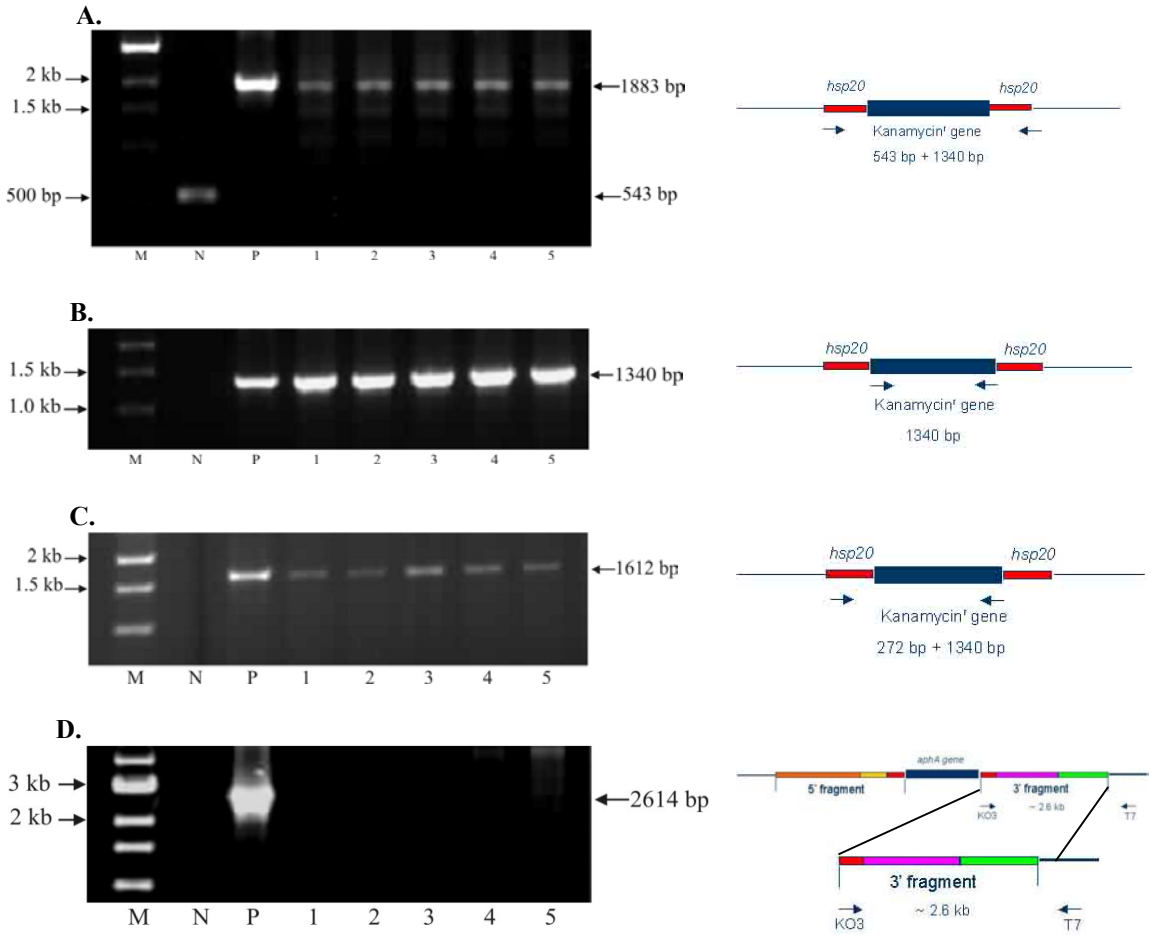


Figure 4.13 PCR identification of kanamycin resistant *H. pylori* clones

The left hand spanel shows the PCR amplification of different fragments using various primers; the right hand panel is a diagrammatic representation of the positions of primers in the targeting vector.

Lanes 1 – 5, the amplifications were based on the genomic DNA of 5 kanamycin resistant *H. pylori* clones (labeled as 1 –5); N, negative control, the amplification was based on *H. pylori* SS1 genomic DNA; P, positive control, the amplification was based on targeting vector DNA; M, 1 kb DNA ladder. Blue arrows indicates the position of primers used.

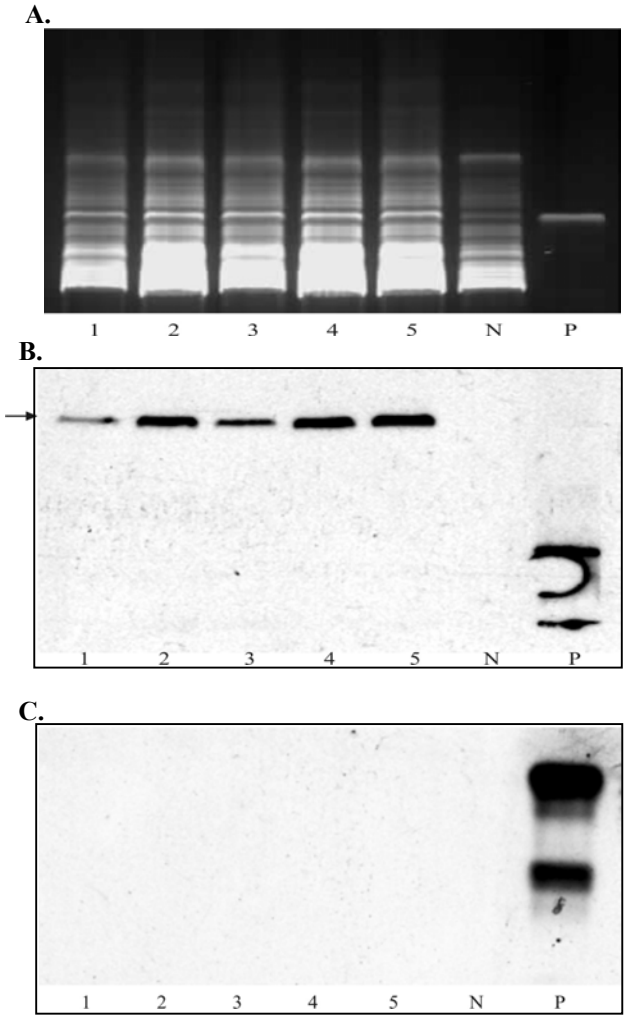


Figure 4.14 Identification of kanamycin resistant *H. pylori* clones by Southern blotting

A, DNA gel image before blotting; **B,** Southern blotting probed with *aphA* gene (1.34kb), the fragment containing *aphA* gene was detected as indicated by arrow; **C,** Southern blotting probed by pBluescript SK plasmid DNA; lane 1 – 5, digested genomic DNA of 5 kanamycin resistant *H. pylori* clones; N, digested genomic DNA of *H. pylori* SS1 serving as a negative control, P, targeting vector DNA serving as a positive control.

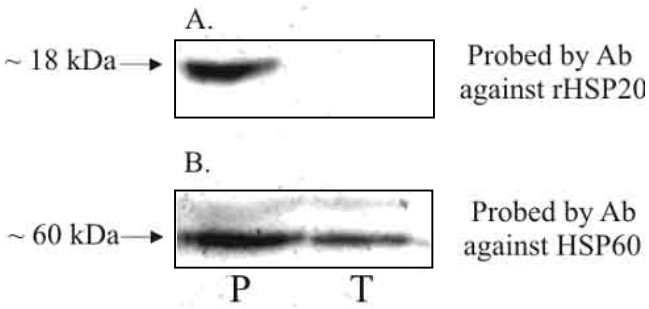


Figure 4.15 Expression of HSP20 in *hsp20*-isogenic *H. pylori* analyzed using Western blotting

5 μg proteins were loaded on SDS-PAGE and blotted on PVDF membrane; specific bands were indicated by arrows. **A,** WB probed with antibody against rHSP20, **B,** WB probed with antiserum against HSP60. P, positive control (AGE of *H. pylori* SS1); T, kanamycin resistant *H. pylori* clone.

4.6 Adherence and colonization study of HSP20 in *Helicobacter pylori*

4.6.1 Adhesion of *H. pylori* to cell lines

The adhesion assay of *H. pylori* to Kato III and AGS cells analyzed using ELISA and flow cytometry showed that the adherence of *hsp20*-isogenic *H. pylori* to cells was 8 – 25% lower than that of wild type *H. pylori* (Table 4.2). However, no significant difference ($p>0.1$) in adherence of the two *H. pylori* (wild type and *hsp20*-isogenic mutant) strains to cell lines was observed *in vitro* based on the statistical analysis.

Table 4.2 Adherence of *hsp20*-isogenic *H. pylori* compared with the wild type

Assays	Bacteria: cell ratio	Adherence to KATO III (%)	Adherence to AGS (%)
		Mutant / WT	Mutant /WT
ELISA	50:1	83.2 ± 1.9	88.3 ± 1.0
	100:1	86.5 ± 0.9	91.6 ± 0.8
	200:1	74.9 ± 1.1	86.3 ± 1.2
Flow Cytometry	50:1	86.7 ± 0.5	86.4 ± 0.5
	100:1	85.6 ± 0.4	83.9 ± 0.6
	200:1	91.6 ± 0.4	88.0 ± 0.5

Mutant: *hsp20*-isogenic *H. pylori*; WT: wild type *H. pylori*. ($p>0.1$)

4.6.2 Analysis of *H. pylori* colonization in mice

4.6.2.1 Microbiological analysis

The results of microbiological analysis of mice biopsy samples are shown in Table 4.3. The growth of pinpoint, transparent colonies on the agar plate are as shown in Figure

4.16A. Using Gram stain, gram-negative bacteria were stained as pink spiral organism (Figure 4.16B). The urease test was positive as indicated by the color change from yellow to pink; oxidase positive bacteria developed deep blue color on the filter paper with oxidase reagent while catalase positive bacteria produced effervescence in the presence of 3% H_2O_2.

4.6.2.2 Histological analysis

Immunohistological stained gastric tissue sections from representative tissues are shown in Figure 4.17. Wild type *H. pylori* inoculated mice revealed brown color, spiral, rod-shaped bacteria on the luminal surface (Figure 4.17B) where bacteria were scattered throughout the corpus and were commonly embedded in mucus layer. However, in the sections of *hsp20*-isogenic *H. pylori* mutant inoculated mice stomach, there was no distinguishable bacterial cell observed and the gastric mucus layer was relatively intact and clear (Figure 4.17C) as negative control of mice stomach (Figure 4.17A) when compared with that of the wild type (Figure 4.17B). The results of histological analysis are as shown in Table 4.3.

4.6.2.3 RT-PCR analysis

The amplified 16s rRNA and urease C gene fragment of *H. pylori* from mice biopsy samples were 390 bp and 294 bp respectively as shown in Figures 4.18A & B. The amplification of GAPDH gene fragment from gastric tissue was 451 bp as shown in Figure 4.18C which was serving as the internal control. The results of RT-PCR analysis of mice biopsy samples are as shown in Table 4.3.

Table 4.3 Analysis of *H. pylori* inoculated mice biopsy samples

H. pylori **inoculated**	**Assays**	**Rate of *H. pylori* detected**			**Total**
		2 weeks	**4 weeks**	**8 weeks**	
Wild Type	Microbiological	5/5	5/5	5/5	15/15 (100%)
H. pylori	Histological	5/5	5/5	5/5	15/15 (100%)
	RT-PCR	5/5	5/5	5/5	15/15 (100%)
hsp20-isogenic	Microbiological	0/5	0/5	0/5	0/15 (0%)
H. pylori	Histological	0/5	0/5	0/5	0/15 (0%)
	RT-PCR	0/5	0/5	0/5	0/15 (0%)

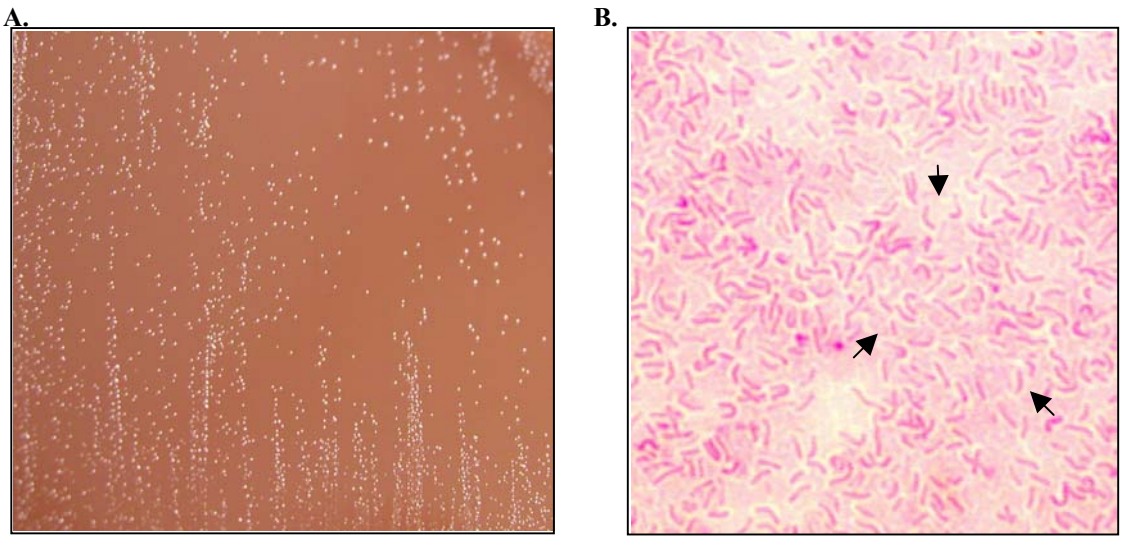


Figure 4.16 Morphological features of *H. pylori*

A, The pinpoint colonies of *H. pylori* on chocolate blood agar plate; **B**, gram staining of a 3-day old *H. pylori* culture (1,000 × magnifications). Pink color of spiral-shaped bacteria indicates that it's gram-negative bacteria (as arrows shown in a very crowded smear).

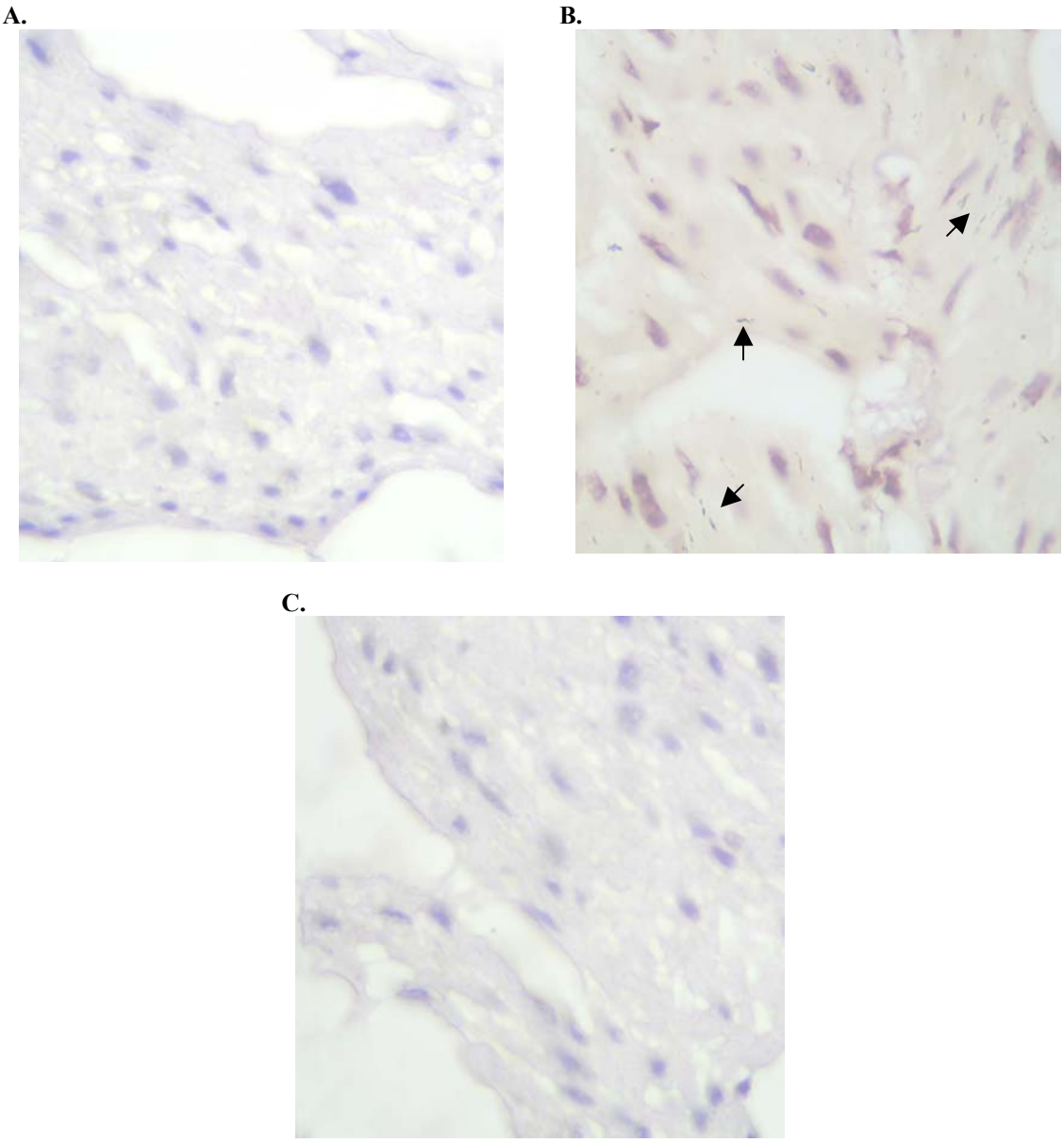


Figure 4.17 Immunohistological detection of *H. pylori* in mice biopsy samples

A, negative control of mice biopsy samples; **B,** wild type *H. pylori* inoculated mice biopsy sample, arrows show the location of *H. pylori*; **C,** *hsp20*-isogenic *H. pylori* inoculated mice biopsy sample. (1000 × magnification).

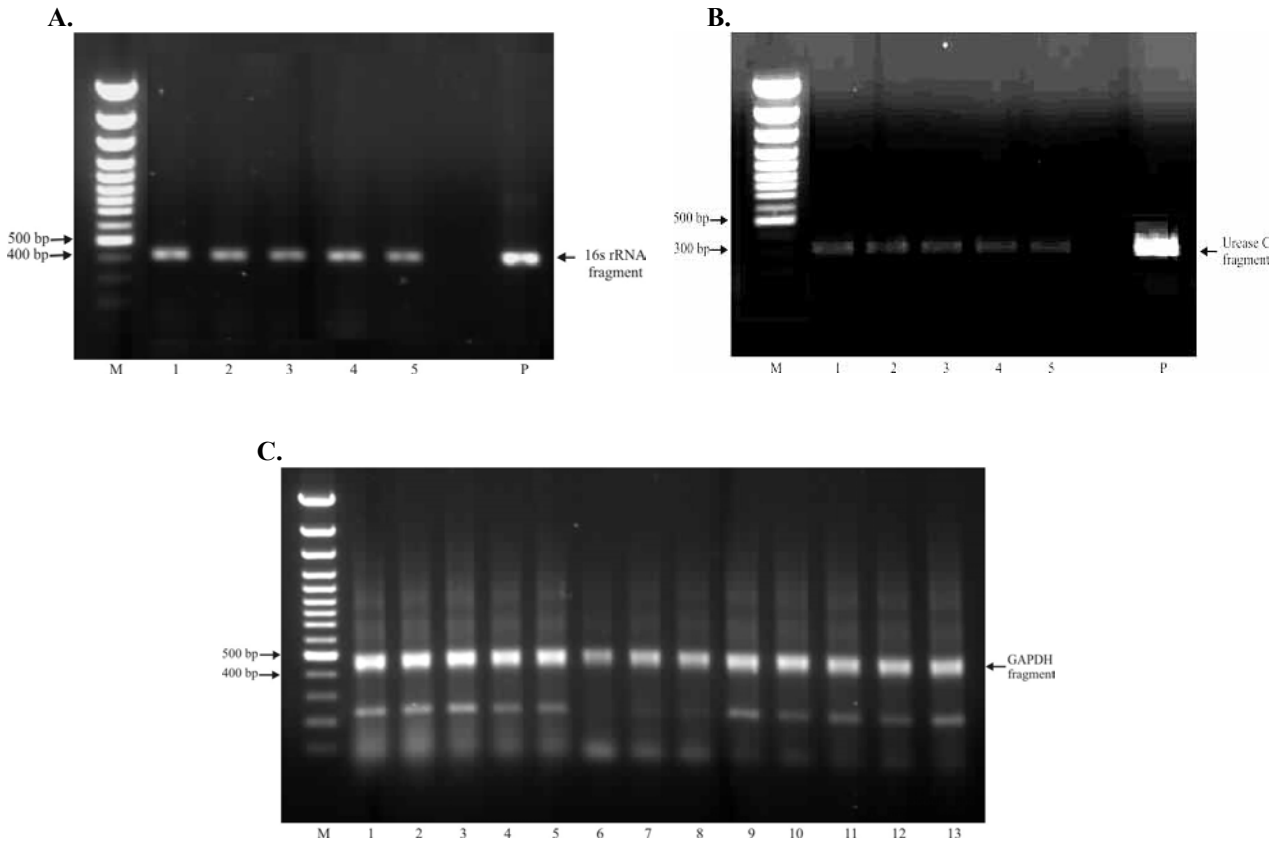


Figure 4.18 RT-PCR analysis of *H. pylori* infected mice biopsy samples

A, Amplification of 16s rRNA gene fragment (390 bp) of *H. pylori*; **B**, Amplification of urease C gene fragment (294 bp) of *H. pylori*; **C**, Amplification of GAPDH gene fragment (451 bp). In A & B, lanes 1 – 5, samples from wild type *H. pylori* infected mice; P, positive control using *H. pylori* SS1 genomic DNA as template. In C, lanes 1 – 3, samples from mice negative controls; lanes 4 – 8, samples from wild type *H. pylori* infected mice; lanes 9 – 13, samples from *hsp20*-isogenic *H. pylori* infected mice. M, 100 base pair DNA ladder.

4.6.2.4 Antibody detection in *H. pylori* inoculated mice

The total antibody against *H. pylori* in serum were detected throughout the course of *H. pylori* infection in mice as monitored using ELISA. The total antibody level in mice inoculated with wild type *H. pylori* was significantly higher ($p<0.01$) than that in the *hsp*20-isogenic *H. pylori* inoculated mice as shown in Figure 4.19A. However, there was no difference in the antibody level against HSP20 in both *H. pylori* inoculated mice as illustrated in Figure 4.19B.

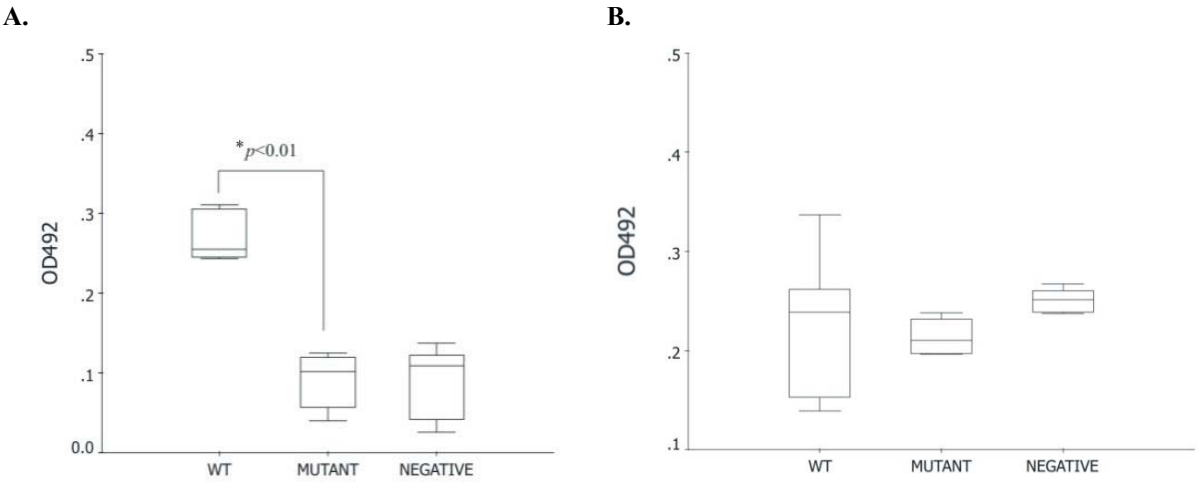


Figure 4.19 ELISA analysis of antibody level in *H. pylori* inoculated mice

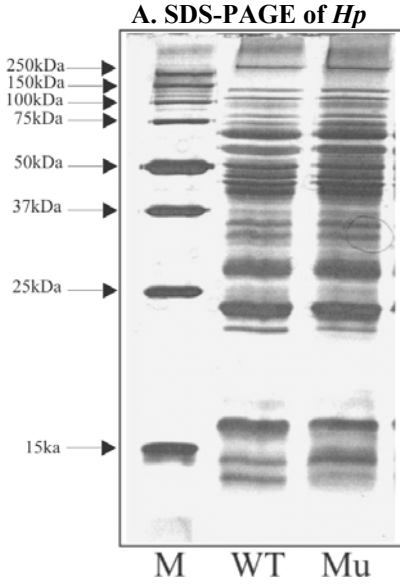
A, Total antibody (IgM + IgG) against *H. pylori* detected in both *H. pylori* inoculated mice; **B**, Antibody level against HSP20 in mice. WT, wild type *H. pylori* inoculated mice; MUTANT, *hsp20*-isogenic *H. pylori* inoculated mice; NEGATIVE, negative control mice fed with BHI broth alone. * The differences between wild type and *hsp20*-isogenic *H. pylori* mutant inoculated mice were compared and statistically significant ($p<0.01$).

4.7 Protein profile of *Helicobacter pylori*

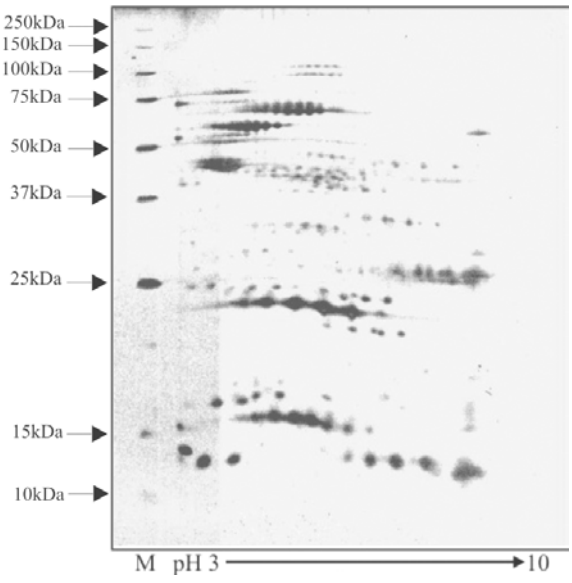
To examine whether disrupted HSP20 would affect the expression of any proteins in *hsp20*-siogenic *H. pylori* mutant, the protein profiles of both wild type and the isogenic mutant *H. pylori* were analyzed on SDS-PAGE and 2D gel electrophoresis (2DE). The

results showed that the protein profiles of the isogenic mutant were visually similar as those of the wild type *H. pylori* on both SDS-PAGE and 2DE (Figure 4.20A, B & C). Interestingly, a clear spot was demonstrated in the Western blotting of wild type 2DE but not that of the isogenic mutant when probed with antibody against rHSP20 (Figure 4.20 D &E).

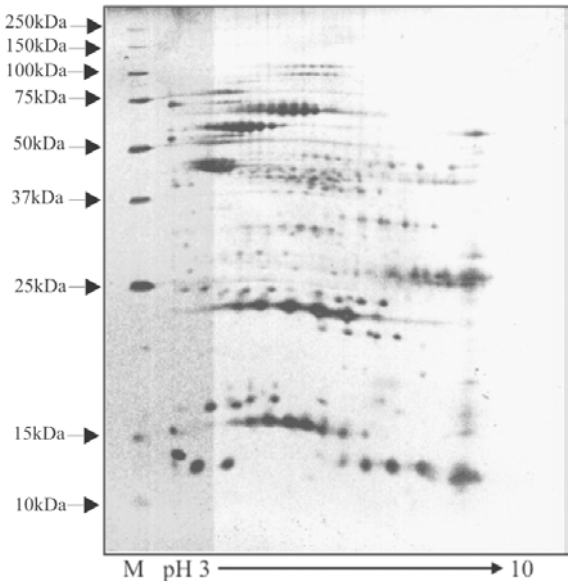
A. SDS-PAGE of *Hp*



B. 2-DE of wild type *Hp*



C. 2-DE of *hsp20*-isogenic *Hp*



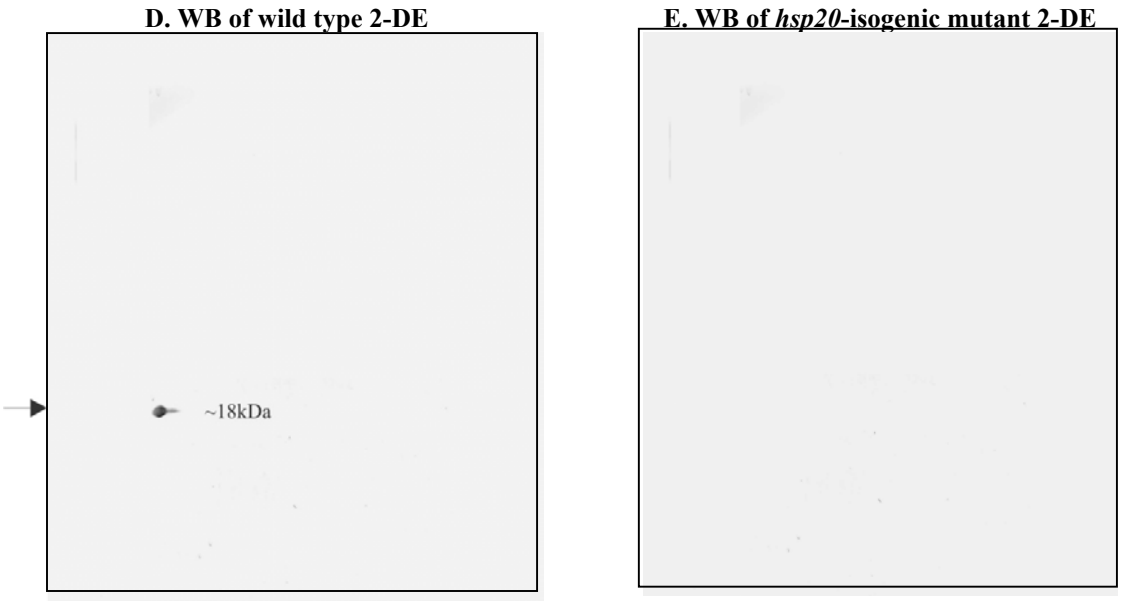


Figure 4.20 Protein profiles of *H. pylori*

A, SDS-PAGE of *H. pylori* total protein extract. WT, wild type *H. pylori*, Mu, *hsp20*-isogenic *H. pylori*;
B, Two dimensional gel electrophoresis of wild type *H. pylori* total protein extract;
C, Two dimensional gel electrophoresis of *hsp20*-isogenic *H. pylori* total protein extract.
M, Prestained Precision Protein Standards (Biorad).

4.8 Functional status of *Helicobacter pylori* adhesins

The DNA sequencing results showed that there were 6, 7 and 7 CT repeats in the signal sequences of *oipA, hopZ* and *sabA* genes respectively (Table 4.4). The CT repeats were consistent in both wild type *H. pylori* and *hsp20*-isogenic *H. pylori* mutant for each gene (Table 4.4). Based on the deduced amino acids in this region, it showed that the open reading frames (ORF) of these three genes are in-frame ("on" status) in both wild type and *hsp20*-isogenic *H. pylori* strains.

RT-PCR showed that all these three genes (*oipA*, *hopZ* and *sabA*) are transcribed in both wild type and *hsp20*-isogenic *H. pylori* strains tested (Figure 4.21A). This further confirms the "on" status of these genes based on the results obtained from DNA

sequencing. However, *babA2* gene was absent in both wild type and the isogenic *H. pylori*. As the internal control, the transcription of 16s rRNA gene was consistent in both wild type and the isogenic *H. pylori* (Figure 4.21B).

Table 4.4 Functional status of *H. pylori* adhesins

Gene	Strain	Partial DNA sequences	Number of CT repeats	Gene status
oipA (HP0638)	**Wild type** *H. pylori*	ATGAAAAAAGCTCTCTTACTAACTCTCTCTCTCTCGTTTTGG M K K A L L L T L S L S F W	6	On
	***hsp20*-isogenic** *H. pylori*	ATGAAAAAAGCTCTCTTACTAACTCTCTCTCTCTCGTTTTGG M K K A L L L T L S L S F W	6	On
HopZ **(HP0009)**	**Wild type** *H. pylori*	ATGAAAAAAACCCTTTTACTCTCTCTCTCTCTCGCTTCATCG M K K T L L L S L S L A S S	7	On
	***hsp20*-isogenic** *H. pylori*	ATGAAAAAAACCCTTTTACTCTCTCTCTCTCTCGCTTCATCG M K K T L L L S L S L A S S	7	On
sabA (HP0725)	**Wild type** *H. pylori*	ATGAAAAAGACAATTCTGCTCTCTCTCTCTCTCGCTTCATCG M K K T I L L S L S L A S S	7	On
	***hsp20*-isogenic** *H. pylori*	ATGAAAAAGACAATTCTGCTCTCTCTCTCTCTCGCTTCATCG M K K T I L L S L S L A S S	7	On

The CT dinucleotide repeats are highlighted in green colour.

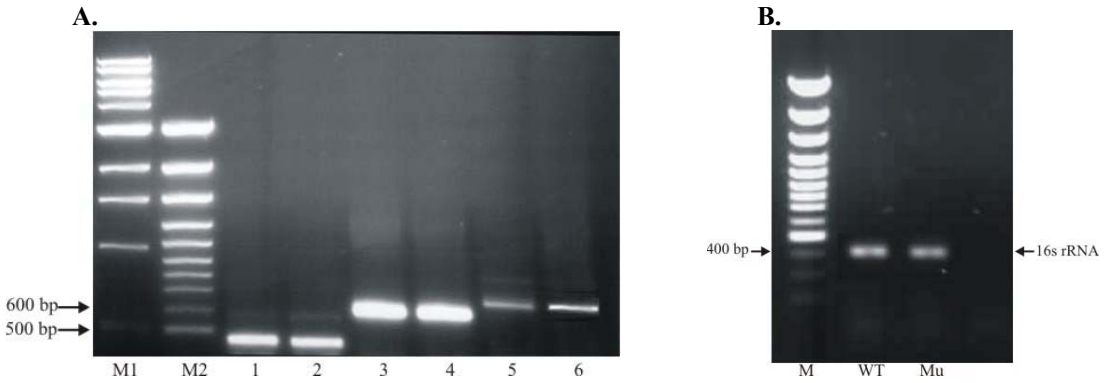


Figure 4.21 RT-PCR analysis of wild type and *hsp20*-iosgenic *H. pylori* adhesins

A, RT-PCR analysis of three adhesins of *H. pylori*; lanes 1 & 2, *oipA* gene fragment (457 bp); lanes 3 & 4, *hopZ* gene fragment (611 bp); lanes 5 & 6, *sabA* gene fragment (643 bp); lanes 1, 3 & 5, RT-PCR amplified based on wild type *H. pylori*; lanes 2, 4 & 6, RT-PCR amplified based on *hsp20*-isogenic *H. pylori*. M1, 1 kb DNA ladder; M2, 100 based pair DNA ladder.

B, RT-PCR analysis of 16s rRNA gene fragment (390 bp); WT, RT-PCR amplified based on wild type *H. pylori*; Mu, RT-PCR amplified based on *hsp20*-isogenic *H. pylori*; M, 100 base pair DNA ladder.

4.9 Analysis of protein interacting with HSP20

4.9.1 Co-immunoprecipitation and Western blotting analysis

A specific band with molecular weight of 100-150 kDa was pulled down by antibody against rHSP20 from the total protein extract of *hsp20* wild type *H. pylori* as shown in Figure 4.22. This specific protein band was only present in the CO-IP with wild type *H. pylori* but absent from *hsp20*-isogenic *H. pylori* protein extract. The same results were replicated under four independent experiments.

This specific protein band was identified as the cytotoxicity associated immunodominant antigen (120 kDa, CagA) of *H. pylori* by mass spectrometry which accession No in NCBI protein database is P55746. The matched peptides and spectrum are shown in Figure 4.23. The same results were obtained in four independent experiments.

Based on the results obtained from CO-IP and protein identification, Western blotting analysis using different antibodies was carried out for CO-IP test. In the Western blotting analysis probed with antiserum against rCagA, a specific band with molecular weight of 100 – 150 kDa was lighted up in the CO-IP protein mixture of wild type *H. pylori* but absent in both *hsp20*-isogenic *H. pylori* and CagA negative *H. pylori* strains (Figure 4.24A). When WB probed with antibody against rHSP20, a specific protein band of ~ 18

kDa was recognized in the CO-IP protein mixture of both wild type and CagA negative *H. pylori* strains but absent in *hsp20*-isogenic *H. pylori* (Figure 4.24B). These results further confirmed the results obtained from protein identification based on MS.

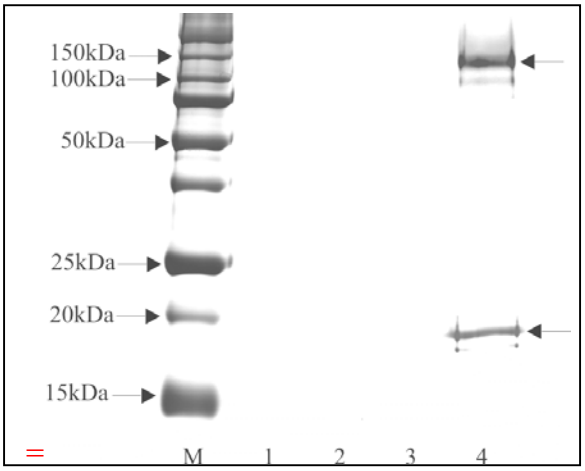


Figure 4.22 SDS-PAGE (12%) analysis of CO-IP

SDS-PAGE of CO-IP with antibody against rHSP20; lane 1, CO-IP of *E. coli* cells with rHSP20 antibody (negative control); lane 2, CO-IP of wild type *H. pylori* proteins with pre-immune rabbit serum (internal negative control); lane 3, CO-IP of *hsp20*-isogenic *H. pylori* proteins with rHSP20 antibody; lane 4, CO-IP of wild type *H. pylori* proteins with rHSP20 Ab. M, prestained Precision Protein Standards (Biorad).

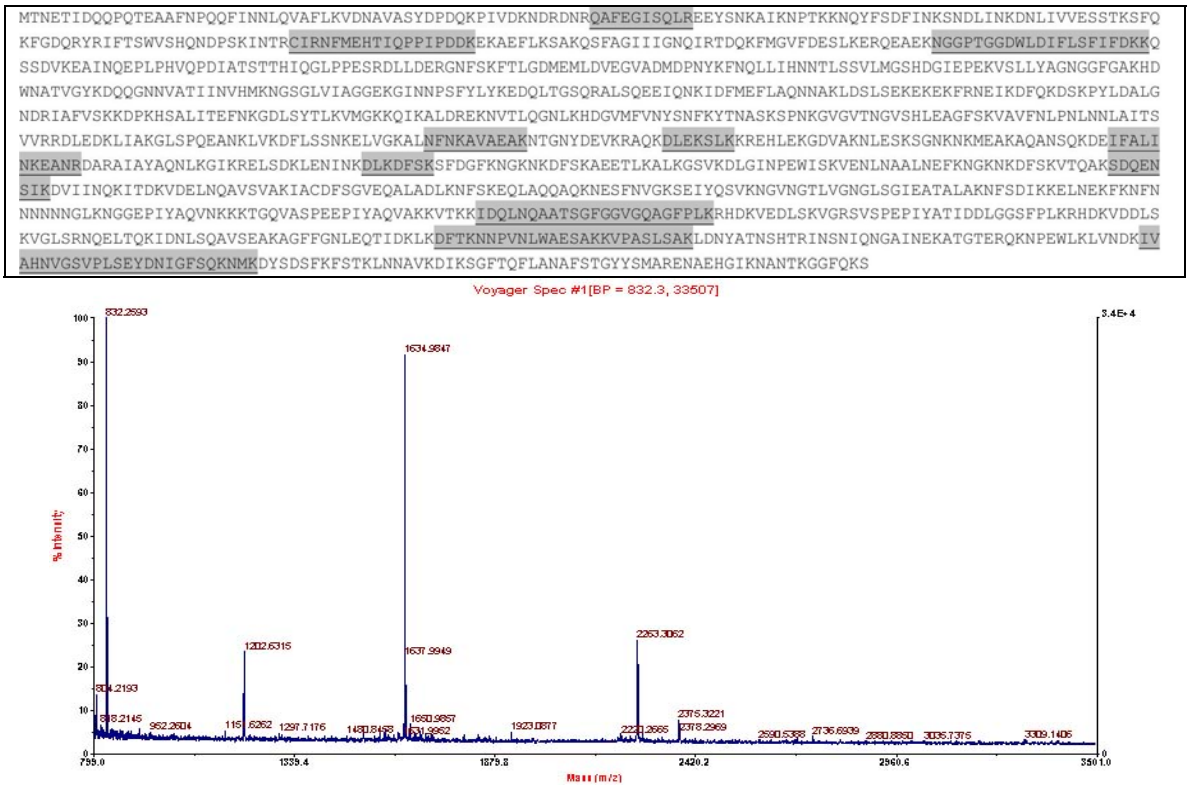


Figure 4.23 Protein identification in CO-IP by MS MALDI-TOF

Upper, Amino acids sequences of CagA (acc. P55746 in NCBI) identified; the matched peptides were in gray color and underlined. **Lower**, spectrum of trypsin digested protein; 11 out of 34 peptides matched with 32% identity.

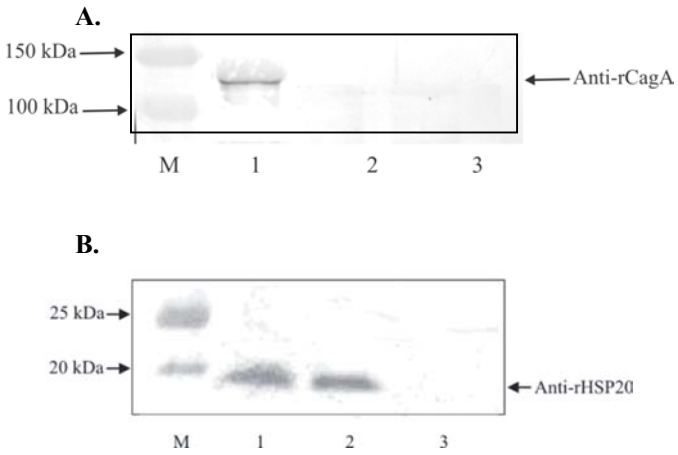


Figure 4.24 Western blotting analysis of CO-IP

A, WB probed with antiserum against rCagA; **B,** WB probed with antibody against rHSP20. Lane 1, CO-IP of wild type *H. pylori*; lane 2, CO-IP of CagA negative *H. pylori*; lane 3, CO-IP of *hsp20*-isogenic *H. pylori*; M, prestained Precision Protein Standards (Biorad).

4.9.2 Transcription of *cagA* in *H. pylori* detected by RT-PCR

The transcription of *cagA* gene in both wild type and *hsp20*-isogenic *H. pylori* cells was detected using RT-PCR analysis. As shown in Figure 4.25A, the fragment of CagA (852 bp) was amplified in both *H. pylori* cells irrespective of ages of culture. As the internal controls, the amplification of 16s rRNA was detected in both *H. pylori* cells as shown in Figure 4.25B while the amplification of *hsp20* gene was only detected in wild type *H. pylori* as shown in Figure 4.25C.

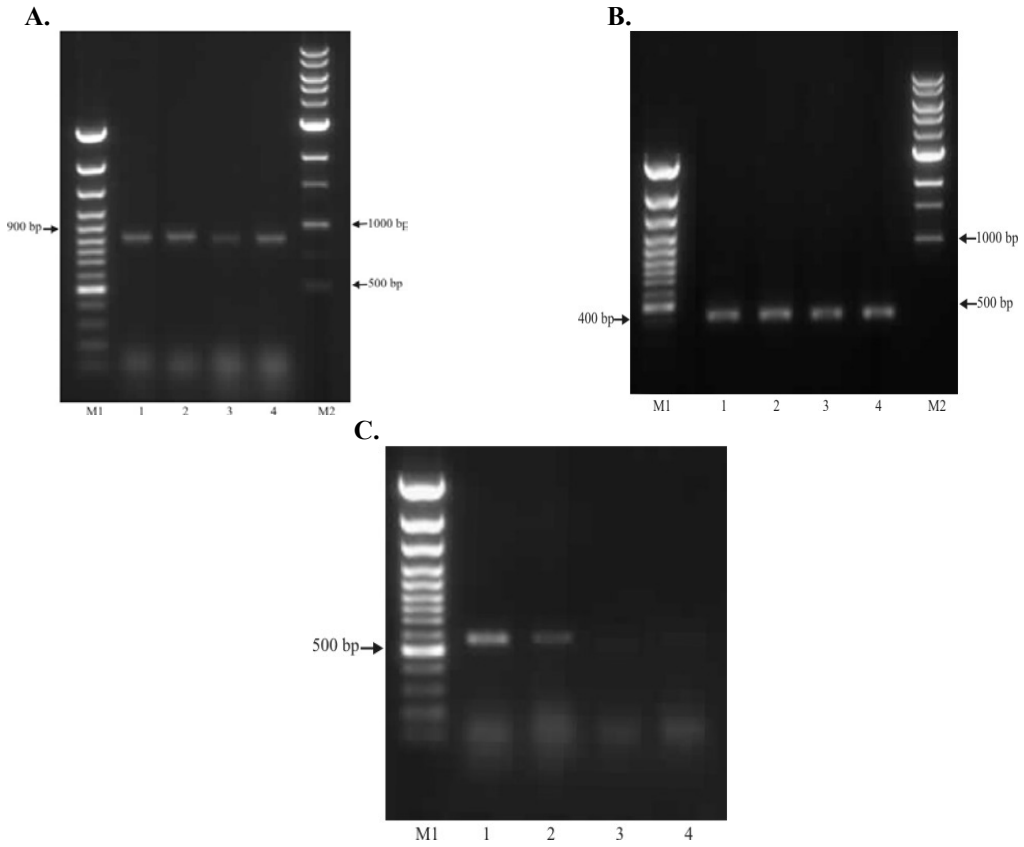


Figure 4.25 *cagA* transcription in *H. pylori* analyzed by RT-PCR

A, Amplification of *cagA* gene fragment (852 bp) in *H. pylori*; **B,** Amplification of 16s rRNA gene fragment (390 bp) **C,** Amplification of *hsp20* gene fragment (543 bp). Lanes 1 & 2, RT-PCR based on 2-day and 3-day old wild type *H. pylori*, respectively: Lanes 3 & 4, RT-PCR based on 2-day and 3-day old *hsp20*-isogenic *H. pylori*, respectively; M1, 100 bp DNA ladder; M2, 1 kb DNA ladder.

4.9.3 Identification of CagA in different *H. pylori* sub-cellular fractions

CagA protein was detected in all fractions extracted (TP, AGE & OMP) from wild type *H. pylori* when probed with antiserum against rCagA (Figure 4.26A upper, lanes 1 – 3). However, CagA was only detected in TP and OMP fractions but absent in AGE fraction of *hsp20*-isogenic *H. pylori* (Figure 4.26A upper, lanes 7 – 9). Furthermore, the intensity of CagA protein band detected in OMP fraction of the mutant is significantly lower than that of the wild type when equal amount of protein was loaded. Using CagA negative strain as a negative control, CagA protein was not detected in all the tested fractions (TP, AGE & OMP) (Figure 4.26A upper, lanes 4 – 6). As an internal control, HSP60 was detected in all test fractions of wild type, CagA negative and *hsp20*-isogenic *H. pylori* strains (Figure 4.26A lower).

In order to determine whether the presence of HSP20 affects the presence of CagA in *H. pylori*, rHSP20 was added into respective *H. pylori* (wild type, the isogenic and CagA negative) cultures. Interestingly, with the addition of rHSP20, CagA protein was detected in all the fractions (TP, AGE & OMP) of both wild type and *hsp20*-isogenic *H. pylori* strains (Figure 4.26B upper, lanes 1 –3 & 7 – 9) using Western blotting. Furthermore, the CagA protein band detected in all fractions of both wild type and the isogenic *H. pylori* showed similar intensity regardless of various concentrations of rHSP20 added (0.1 – 1 μg). There was no CagA detected in any of the fractions of CagA negative *H. pylori* (Figure 4.26B upper, lanes 4 – 6). As the internal control, HSP60 was also detected in every fraction of wild type, the isogenic and CagA negative *H. pylori* strains (Figure 4.26B lower).

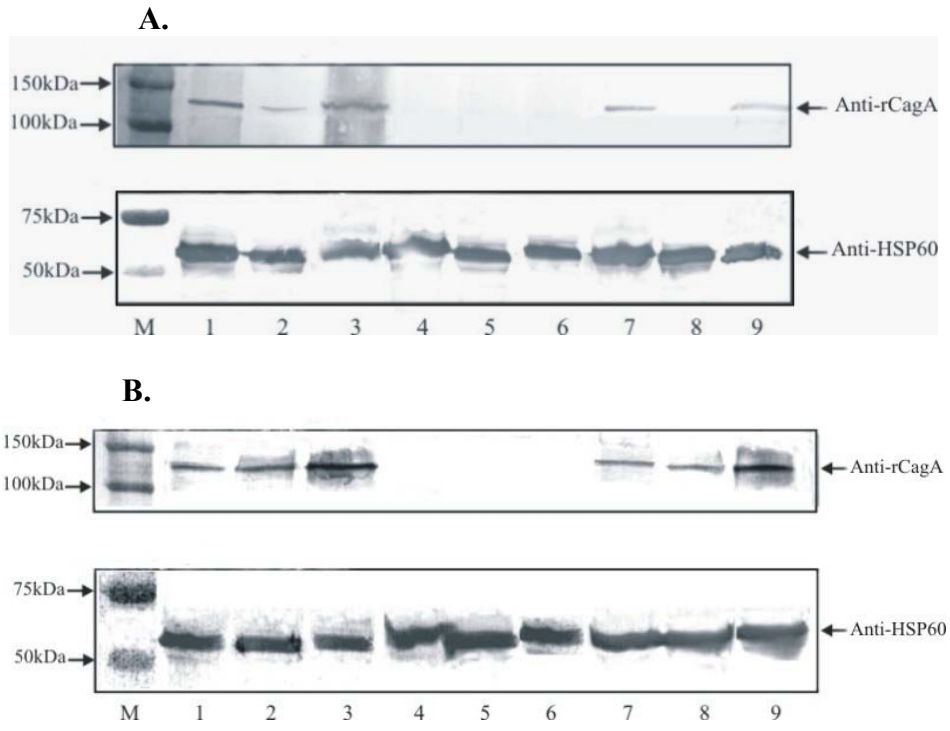


Figure 4.26 Presence of CagA in different *H. pylori* sub-cellular fractions

A, *H. pylori* grown under normal conditions (broth culture); **B**, representative of CagA partition in *H. pylori* grown with the addition of rHSP20 for 24 hours. Lanes 1, 4 & 7, total protein (TP) extract; lanes 2, 5 & 8, acid glycine extract (AGE), lanes 3, 6 & 9, outer membrane protein (OMP) extract. Lanes 1 –3, protein extracts of wild type, lanes 4 – 6, protein extracts of CagA-negative; lanes 7 – 9, protein extracts of *hsp20*-isogenic mutant. M, prestained Precision Protein Standards (Biorad). An amount of 10 μg protein was loaded per lane in 10% SDS-PAGE.

4.9.4 Antibody against CagA in *H. pylori* infected mice

The antibody levels against CagA in the isogenic mutant infected mice detected were significantly lower ($p<0.05$) than that of the mice infected with wild type *H. pylori* (Figure 4.27A). However, the antibody against Le (X) and Le (Y) were insignificant between the mice infected with *H. pylori* wild type and the isogenic mutant (Figure 4.27B & C). As bacterial Lewis antigen has been reported to be involved in the adhesion of *H. pylori* (Edwards *et al*., 2000), the presence of antibodies against Le (X) and Le (Y) is

suggested to be induced during the adhesion of *H. pylori* prior to the releasing of virulence factors.

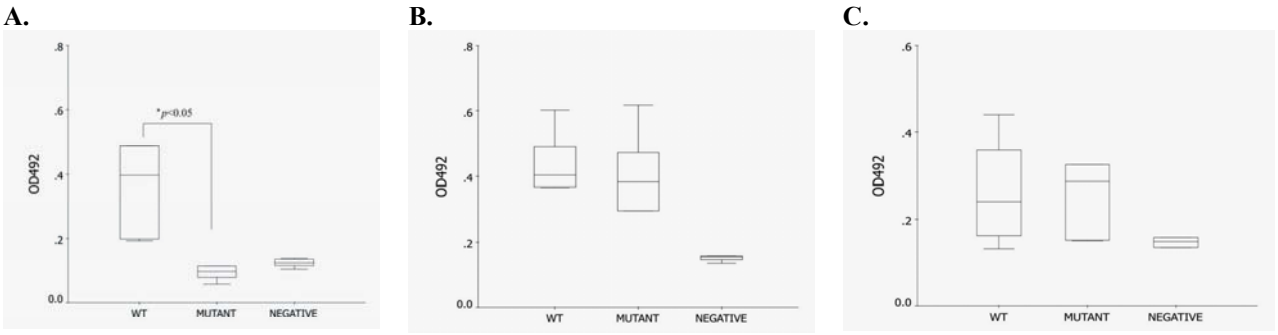


Figure 4.27 Antibody against CagA detected in *H. pylori* inoculated mice

A, antibody against CagA detected in *H. pylori* infected mice. **B**, antibody against Le (X) antigen in *H. pylori* infected mice; **C**, antibody against Le (Y) antigen in *H. pylori* infected mice. WT, wild type *H. pylori* inoculated mice; MUTANT, *hsp20*-isogenic *H. pylori* inoculated mice; NEGATIVE, negative control mice fed with BHI broth alone. * The difference between wild type *H. pylori* inoculated mice and mutant was statistically significant (p<=0.05).

4.10 Use of HSP20 for the epidemiological study in *Helicobacter pylori*

4.10.1 Nucleic acid sequences analyses

The nucleic acid sequences of *hsp20* from 227 *H. pylori* isolates showed an open reading frame of 540 bp with neither deletion nor insertion. However, upon comparison there were a total of 219 polymorphic sites observed which were scattered in the whole gene fragment, showing high level of synonymous sequence variations and most of the nucleotide substitutions (57.99%) were at the third codon position. The (G+C)% content of all *hsp20* sequences analyzed ranged from 41.25% to 44.57% with an average of 43.12%. The percentage of the differences between pairs of strains at synonymous nucleotide positions (*Ks*) was 15.9% and 1.25% at the non-synonymous positions (*Ka*).

The ratio of *Ks/Ka* was 12.65 that is lower than that of some housekeeping genes (e.g., *atpD*: 82.5; *scoB*: 37.7; *glnA*: 22 and *recA*: 20.3) as reported by Maggi *et al*. (2001).

4.10.2 Phylogenetic analysis

The dendrogram as shown in Figure 4.10 was generated based on the analyses of nucleic acid sequences of *hsp20* from 225 tested strains and the 2 established strains (26695 & J99) which genomic DNAs have been sequenced (Tomb *et al*., 1997; Alm *et al*., 1999). Using PHYLIP and ML algorithms, the inferred taxonomic distance between different *H. pylori* strains was arrived as illustrated in Figure 4.28. Two major clusters (A & B) were observed: a larger group A and a smaller group B. The isolates in group A shows high similarity in its DNA sequences and further bifurcates into two subgroups, namely A_1 and A_2. Interestingly, subgroup A_1 and group B were mainly from Asian and non-Asian origins, respectively. However, subgroup A_2 comprises a mix of *H. pylori* isolates of Asian and non-Asian origins. In group A_1 (n=119), all the isolates except for CR10498 (Costa Rica), SJM1 and SJM14 (Peruvian) and Cau1026 (an isolate from an European visitor in Singapore) are entirely from Asian countries. There were 34 Asian strains and 36 non-Asian strains that made up subgroup A_2 (n=70). The remainder (n=35) including four Singapore strains (Sin1059, 541, 1134 & 1024, all were isolates from Malay ethnic origin) constituted group B. There were 3 strains (J1186, HK77 and SJM19) which were not aligned to any of the groups. The percentage of distribution shows that Asian isolates are present in 96.64% (group A_1; 115/119), 48.57% (group A_2; 34/70) and 11.43% (group B; 4/35) while non-Asian isolates are present in 3.36% (group A_1), 51.43% (group A_2) and 88.57% (group B).

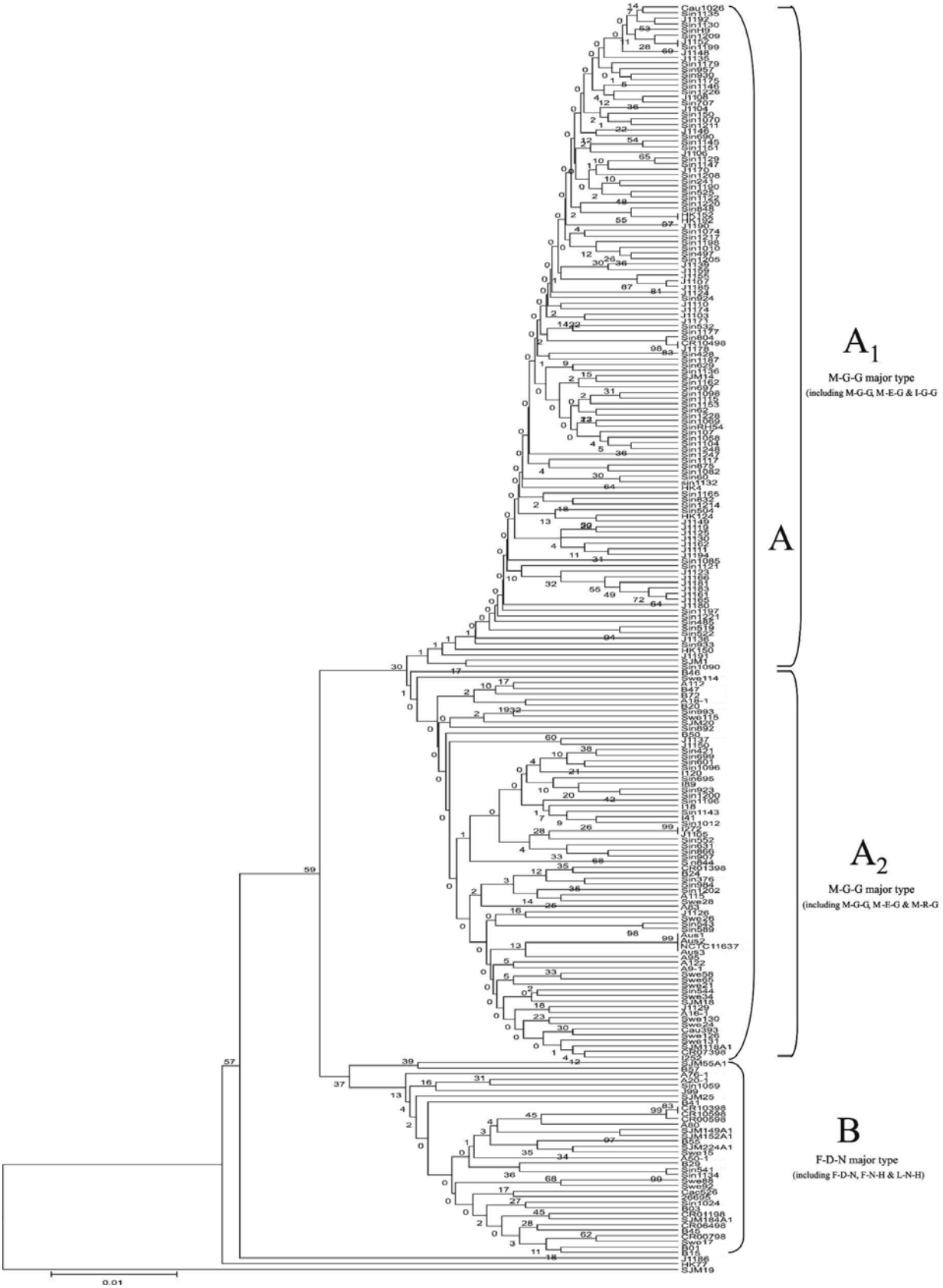

A
A1
M-G-G major type
(including M-G-G, M-E-G & I-G-G
A2
M-G-G major type
(including M-G-G, M-E-G & M-R-G
B
F-D-N major type
(including F-D-N, F-N-H & L-N-H)
0.01

Figure 4.28 The phylogenetic analysis of the 227 *H. pylori* isolates based on *hsp20* DNA sequences

Isolates from **A**: Lithuania; **Aus**: Australia; **B**: Span; **Cau**: other Singapore isolates; **CR**: Costa Rica; **HK**: Hong Kong; **I**: India; **J**: Japan; **Sin**: Singapore; **SJM**: Peru; **Swe**: Sweden. The groups are indicated as A (Asian) & B (non-Asian). The bootstrap replicates are shown at the nodes, the scale bar represents the substitution rate per site. PHYLIP (version 3.6) and ML algorithm were used to conduct the analysis.

The observation was further confirmed by the estimated DNA divergence as shown in Table 4.5. The nucleotide divergence (*D*) within group A_1 (2.43%), A_2 (3.38%) or group B (3.69 %) was relatively lower than the *D* value between groups. The divergence between group A_1 and B (6.03 %) was the highest followed by A_2 and B (5.29 %) or A_1 and A_2 (4.34%). The value of estimated *Ks* between different groups (Table 4.5) from high to low was in the order of A_1 vs. B (0.212); A_2 vs. B (0.190) and A_1 vs. A_2 (0.185). Furthermore, it is noted that the divergence differences between different *hsp20* gene groupings were significantly lower when compared with the divergence between *vacA* alleles m1 and m2 (24.9%) or the *Ks* between m1 and m2 (0.46) as reported by Atherton *et al.* (1999).

Table 4.5 Comparison of DNA polymorphism between geographical groups

Comparisons	*D* (%)	*Ks*	*Ka*	Ratio of *Ks/Ka*	Reference
A_1 vs. A_1	2.43 ± 0.06	0.094	0.006	15.6	This study
A_2 vs. A_2	3.38 ± 0.09	0.148	0.005	29.6	This study
B vs. B	3.69 ± 0.19	0.152	0.009	16.9	This study
A_1 vs. A_2	4.34 ± 0.11	0.185	0.009	20.5	This study
A_1 vs. B	6.03 ± 0.17	0.212	0.024	8.3	This study
A_2 vs. B	5.29 ± 0.17	0.190	0.019	10	This study
vacA m1 vs. m2	24.9%	0.46	0.246	1.9	Atherton *et al*. 1999
atpD	-	-	-	82.5	Maggi *et al*. 2001
scoB	-	-	-	37.7	Maggi *et al*. 2001
glnA	-	-	-	22	Maggi *et al*. 2001
recA	-	-	-	20.3	Maggi *et al*. 2001

D: percentage of the average number of nucleotide substitutions per site; *Ks*: the mean differences between pairs of strains at synonymous nucleotide position; *Ka*: the mean differences between pairs of strains at non-synonymous nucleotide position.

4.10.3 Amino acid sequences analyses

The corresponding amino acid sequences of HSP20 from 227 *H. pylori* isolates were deduced from DNA sequences showed a total number of 51 substitutions. Of these, 79% of the amino acid substitutions belong to the same polarity group, e.g. from polar to polar or from hydrophobic to hydrophobic. The remaining 21% were substituted between different groups, e.g. switching between polar and hydrophobic.

There are seven types of substitutions observed at 14^{th} - 16^{th} amino acid residues. These sequences were based on *H. pylori* J99 which substitutions is F-D-N at 14^{th} –16^{th} amino acids, *H. pylori* 26695 for L-N-H, Singapore RH54 for M-G-G, Swedish 58 for M-E-G, Japanese GS11 for I-G-G, Swedish 24 for M-R-G and Swedish 88 for F-N-H. The

substitutions corresponding to positions 14th – 15th – 16th were: M-G-G (~76%), M-E-G (~4%), M-R-G (~0.4%), I-G-G (~3%) constituting the M-G-G cluster while the F-D-N cluster comprises F-D-N (~13.6%), L-N-H (~2.2%) and F-N-H (~0.8%). The substitution linkages were more diverse among the non-Asian group (M-G-G, F-D-N, L-N-H, M-E-G, F-N-H & M-R-G) than the Asian group (M-G-G, I-G-G, M-E-G & F-D-N). Interestingly, substitution linkage (M-G-G) predominates among the Asian group (91%, 143/158) while the F-D-N substitution linkage is found more frequently in *H. pylori* isolates obtained from non-Asian countries (37.5%, 27/69) (Tables 4.6). Among the seven observed substitution linkages, M-G-G is the most prevalent type among all the isolates tested (76%, 172/227).

Table 4.6 Summary of substitutions at 14th –16th amino acids sequences of HSP20

Origins		Substitutions							Total No
		M-G-G	**M-E-G**	**I-G-G**	**M-R-G**	**F-D-N**	**L-N-H**	**F-N-H**	
Asian Countries	**Singapore** (103)								
	Chinese	66	1	3					
	Malay	8				4			
	Indian	18							
	Others*	2					1		
	Hong Kong (6)	6							
	Japan (43)	37	2	4					
	India (6)	6							
	Sub-total No	143	3	7		4	1		158
non-Asian Countries	**Peru** (12)	5				6	1		
	Costa Rica (9)	3				5	1		
	Sweden (16)	7	4		1	2		2	
	Spain (14)	6				7	1		
	Lithuania (12)	7	1			4			
	Australia (4)	1	1			2			
	US (26695)						1		
	British (J99)					1			
	Sub-total No	29	6		1	27	4	2	69
	Total No	172	9	7	1	31	5	2	227

The number of strains obtained from each country is indicated in parenthesis. * These 3 Caucasians are visitors from Western countries living in Singapore (labeled as Cau393, Cau526 and Cau1026).

Interestingly, substitutions at 14th – 16th amino acid residues (Table 4.7) show that significantly more patients with PUD (peptic ulcer disease) harbored *H. pylori* isolates with M-G-G substitution cluster (M-G-G, M-E-G, M-R-G and I-G-G) while those patients with NUD (non-ulcer dyspepsia) possessed *H. pylori* strains with F-D-N substitution cluster (F-D-N, L-N-H and F-N-H). Based on the disease outcome of 195 *H. pylori* strains in this study, the odds ratio (OR) of M-G-G cluster for PUD was calculated. It was shown that M-G-G has an OR of 4.27 in its association with PUD as compare to F-D-N that is positively associated with NUD (Table 4.7).

Table 4.7 Summary of substitutions and the disease status of *H. pylori* isolates

	Type of substitutions	Diseases status		Odds Ratio
		PUD	**NUD**	**(OR)**
Number of isolate & (Percentage)	**M-G-G**	103 (66%)	53 (34%)	For PUD: 4.27
	M-G-G cluster	112 (65%)	60 (35%)	
	F-D-N	6 (33%)	12 (67%)	
	F-D-N cluster	7 (30%)	16 (70%)	

PUD: gastric ulcer & duodenal ulcer; NUD: gastritis & non-ulcer dyspepsia.
M-G-G cluster (M-G-G, M-E-G, M-R-G & I-G-G) is positively associated with PUD.
F-D-N cluster (F-D-N, L-N-H & F-N-H) is positively associated with NUD.

Similarly, interesting findings were observed in regards to the substitutions at 14th – 16th amino acid residues and geographical groupings. The predominant substitution is M-G-G that comprises 172/227 isolates (Table 4.6). Of these, 109 and 61 isolates with M-G-G substitution are located in group A_1 and A_2, respectively (Table 4.8). It shows that group A_1 is predominately the M-G-G substitution with Asian origin while group A_2

comprises a mix of M-G-G substitution of Asian and non-Asian origins. The other substitutions, I-G-G (n= 7) is only found in Asian isolates and strictly distributed in group A_1; M-E-G (n=9) substitution are distributed in both group A_1 and A_2 while the substitution M-R-G with only a single strain of Swe24 is located in group A_2. In contrast, F-D-N substitution was found in 31/227 isolates (Table 4.6). F-D-N is the prevalent substitution type in group B (28/35) (Table 4.8). It is notable that except two F-D-N substitution (Aus3 and NCTC11637) clustered in group A_2, the rest of F-D-N substitution cluster (F-D-N, L-N-H and F-N-H) are located in the group B including four Singapore isolates (Sin1059, 541, 1134 & 1024, all were isolates of Malay ethnic origin).

Table 4.8 The distribution of various substitutions in geographical groupings

	A_1 (n=119)	**A_2 (n=70)**	**B (n=35)**
	Asian (115) Non-Asian (4)	Asian (34) Non-Asian (36)	Asian (4) Non-Asian (31)
M-G-G Cluster (n=187)	M-G-G: 109 M-E-G: 3 I-G-G: 7	M-G-G: 61 M-E-G: 6 M-R-G: 1	-
F-D-N Cluster (n=37)	-	F-D-N: 2	F-D-N: 28 L-N-H: 5 F-N-H: 2

A1: cluster of Asian origin; A2: cluster of Asian and non-Asian origins; B: cluster of non-Asian origin.

4.11 Protein structure prediction of HSP20

4.11.1 HSP20 protein structure prediction

The predicted HSP20 protein structure shows four α helixes, ten β sheets and 14 turns (Figures 4.29). The four α helixes, two at each side, flank the central core of ten β sheets that are concentrated in the middle. All the α helixes and β sheets are evenly distributed on both sides forming a symmetrical structure. The secondary structure elements were compared with two homologues as shown in Table 4.9.

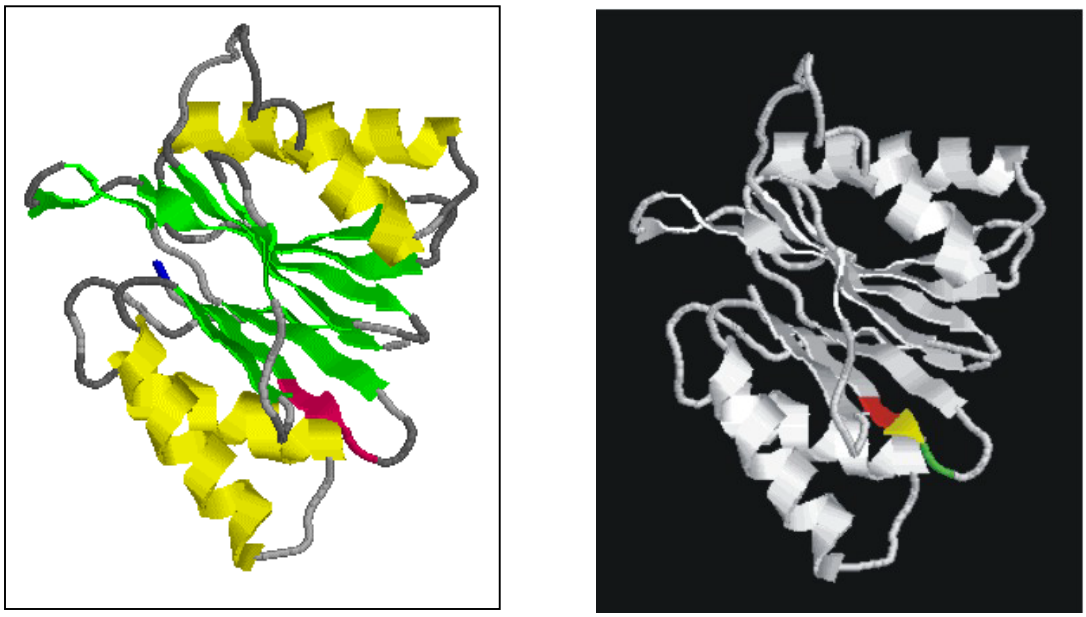


Figure 4.29 The 3-D structure of HSP20 (HP0515) protein predicted by homology modeling

A, The predicted 3-D structure of HSP20 shows four α helixes (yellow), ten β sheets (green) and 14 turns (gray). The starting point of β sheets is highlighted in blue; the 14th to 16th amino acid residues is highlighted in red. **B,** The position of 14th –16th amino acid residues are highlighted in color: 14th (red), 15th (yellow) and 16th (green). SWISS-MODEL server (http://www.expasy.org/swissmod/) was used for the homology modeling.

Table 4.9 Comparison of the Secondary Structure of HSP20 related protein species

Protein species	Secondary structure		
	α Helices	**β Sheets**	**Turns / coils**
HslV (*H. influenzae*)	**4** (10-14 AA)	**10** (3-12 AA)	**12** (2-10 AA)
HslV *(E. coli)*	**5** (3-17 AA)	**10** (3-13 AA)	**14** (1-7 AA)
HSP20 *(H. pylori)*	**4** (10-14 AA)	**10** (3-12 AA)	**14** (2-10 AA)

4.11.2 Structure comparison of substitutions at 14^{th} – 16^{th} amino acid residues

The amino acids of 14^{th} – 16^{th} are located at the end of the first β sheet that displays on the surface of predicted HSP20 protein structures (Figures 4.29 & 4.30). Interestingly, based on the obtained 3-D structure, the seven different substitution linkages (M-G-G, I-G-G, M-E-G, M-R-G, F-D-N, L-N-H and F-N-H) do not affect the protein conformation in the 3-D model predicted (Figure 4.31).

The 3 amino acids variations of 14^{th} – 16^{th} is unique in *H. pylori* HSP20 species and is absent in both HslV of *E. coli* and *H. influenzae* as shown in the alignment of three homologues in Figure 4.32.

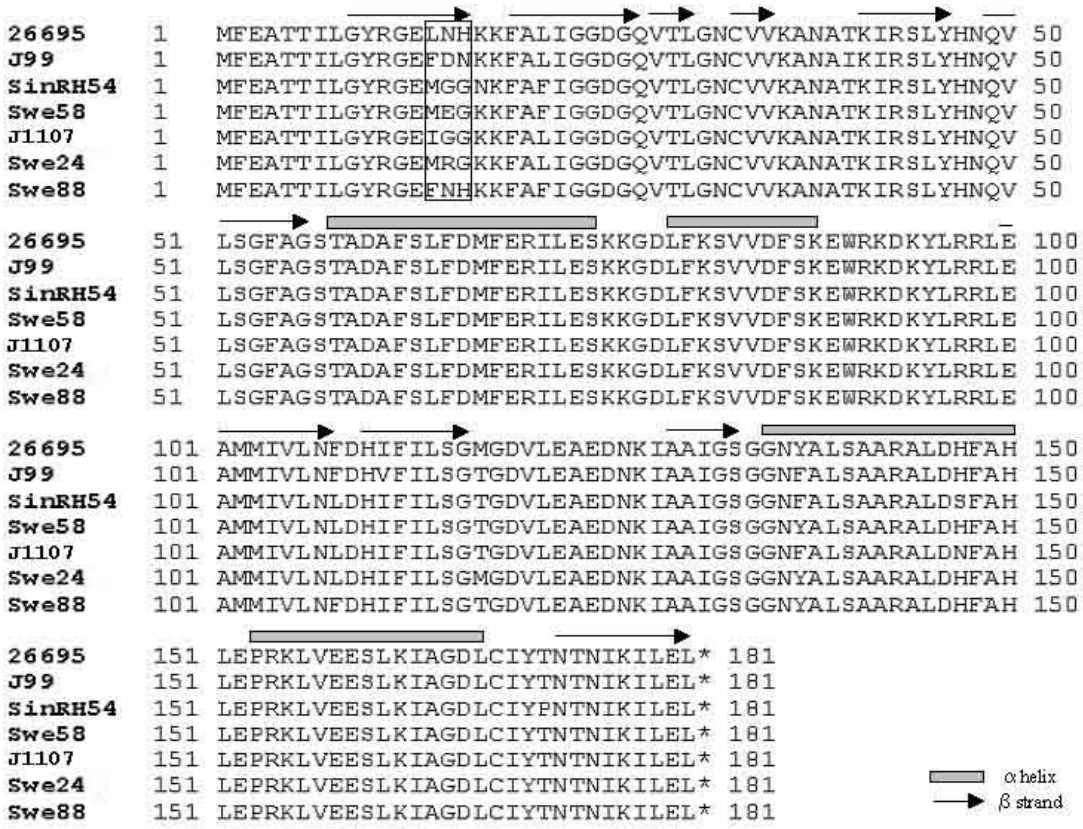


Figure 4.30 The predicted secondary structure of HSP20 (HP0515) protein

The position of 3-amino-acid substitution linkages (14^{th} – 16^{th} amino acids) is framed in the box.

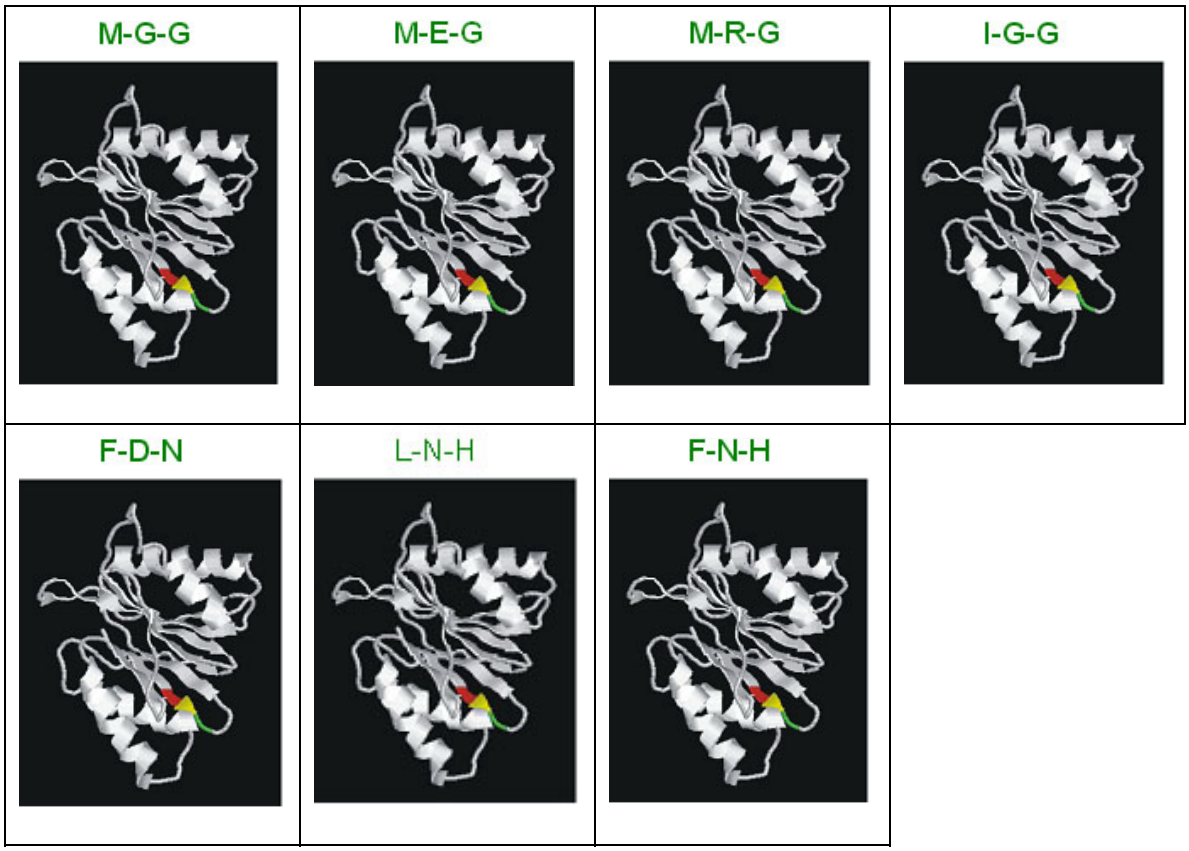


Figure 4.31 The predicted 3-D structure of HSP20 with different substitutions at 14th – 16th amino acid residues

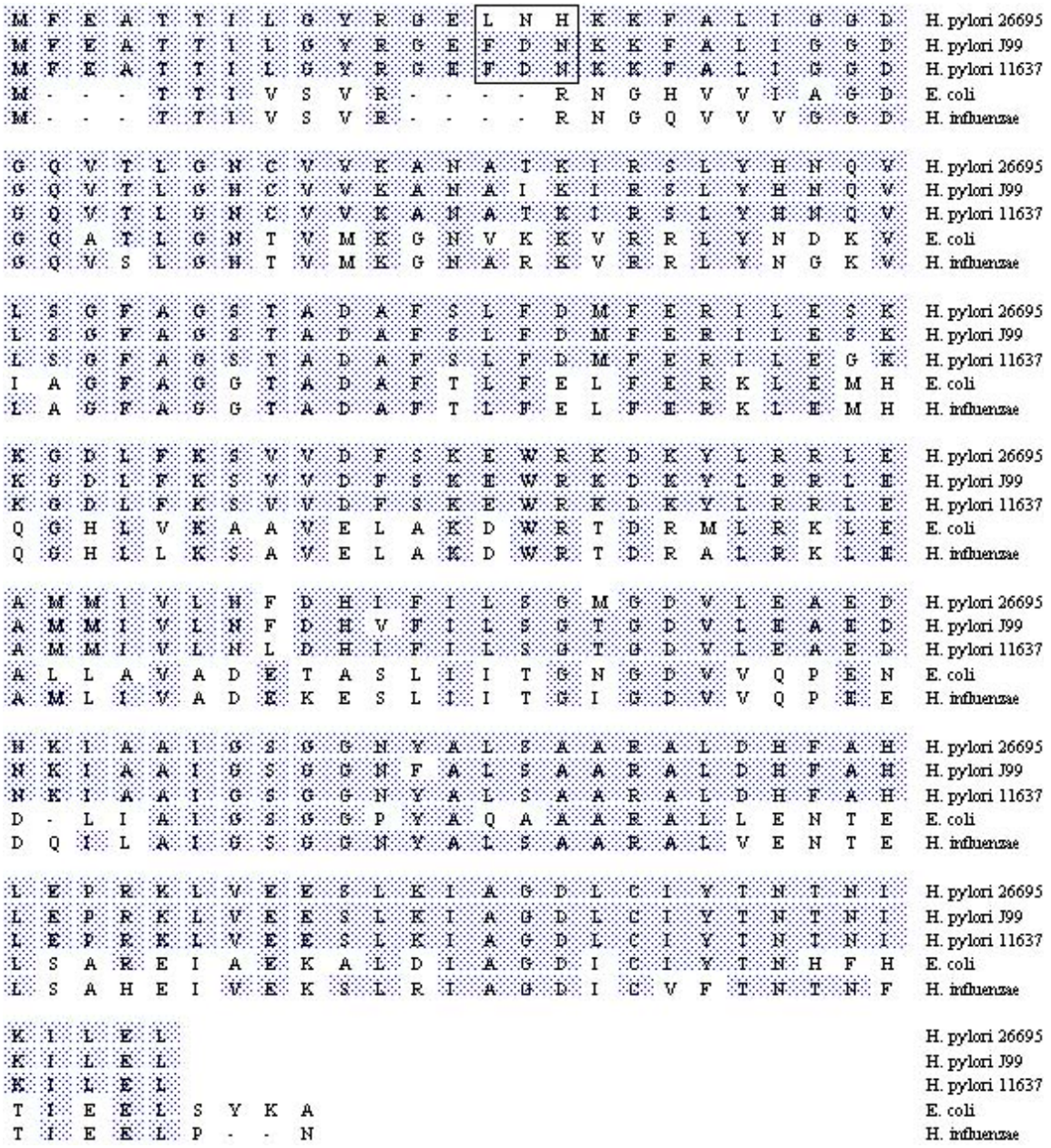


Figure 4.32 The alignment of amino acid sequences of HSP20 and homologues from other bacterial species

The 3-amino-acid substitution linkage (14^{th} – 16^{th} amino acids) is framed in the box. The identical amino acids are highlighted in shadow.

5. DISCUSSION

5.1 Similarity between HSP20 and its homologue – HslV

The amino acid sequence of HSP20 of *H. pylori* 11637 shows 96% and 97% identity to HslV of *H. pylori* strains 26695 (Tomb *et al*., 1997) and J99 (Alm *et al*., 1999), respectively. It also shows 49% identity with HslV of *E. coli* (Sousa *et al*., 2002). Its homologue, HslV was determined as a peptidase that is a component of ATP-dependent protease (HslUV) in *E. coli* (Yoo *et al*., 1996). Based on the annotation of open reading frame by Tomb *et al*. (1997), HslU of *H. pylori* shows 98% identify to its homologue of *E. coli* (Tomb *et al*., 1997) whereas HslV of *H. pylori* merely shows 49% identify to its homologue. The lower percentage of similarity between HslV (HSP20) of *H. pylori* and its homologue as compared with that of HslU implies that HslV (HSP20) of *H. pylori* may be functionally different from its homologues in other bacteria (e.g. *E. coli*).

5.2 Localization of HSP20 in *H. pylori*

5.2.1 Using Western blotting and immuno-gold TEM

Using Western blotting and the antibody against rHSP20 on different *H. pylori* sub-cellular fractions (Figure 4.6), a specific and intensive protein band of ~18 kDa showed up clearly in the membrane and membrane associated protein fraction. These findings indicate that HSP20 is mainly distributed on the cell surface fraction of *H. pylori*.

The surface localization of HSP20 in *H. pylori* was further confirmed by the immuno-gold labeled TEM where both 10 nm and 20 nm of ProteinA-gold particles were shown mainly attached to the cell membrane or the remnants of cell membrane of *H. pylori* (Figure 4.9A & B). These findings strengthen the results obtained from Western blotting analyses verifying that HSP20 is mainly localized on the cell surface of *H. pylori*.

It was noted that HSP20 when expressed as a His-tag fused recombinant protein *in E. coli*, the additional 27 amino acids upstream HSP20 sequences added another about 3 kDa resulted in a recombinant HSP20 (rHSP20) with molecular weight (MW) of about 23 kDa. However, the observed MW of native HSP20 was about18 kDa (Figure 4.6A & 4.7), according to the amino acids sequences, the predicted MW of HSP20 is 20 kDa (Tomb *et al*., 1997). The major differences on MW variation of HSP20 between predicted and the actual observation of about 2 kDa is likely due to the preparation of protein extract or unknown factors in SDS-PAGE.

5.2.2 Surface localization of HSP20 in *H. pylori*

The surface localization of HSP20 in *H. pylori* identified in this study is different from the prediction based on the sequence similarity recommended in SWISS-PROT (available at http://www.isb-sib.ch), where the sub-cellular localization of HslV (HSP20) of *H. pylori* was cytoplasmic. The differences in protein localization between *H. pylori* and other bacteria are also observed in a number of protein species e.g., HSP60, HSPA, urease, catalase, superoxide dismutase (SOD) that are found exclusively within cytoplasm in other bacteria but identified as surface associated in *H. pylori* (Hawtin *et al*., 1990; Phadnis *et al*., 1996; Mori *et al*., 1997; Vanet & Labigne, 1998).

Although HSP20 of *H. pylori* is a homologue of HslV of *E. coli*, the similarity between them is relatively lower than that between the homologues of HslU or other heat shock proteins e.g., HSP60 or HSP70 in which the identities are as higher than as 60% or above (Tomb *et al*., 1997). The low percentage of similarity between HSP20 and HslV of *E. coli* and the surface localization of HSP20 may imply the different role of HSP20 in *H.*

pylori and further implies the presence of different function between HSP20 from HslV of *E. coli*.

5.2.3 Possible mechanism for surface localization of HSP20 in *H. pylori*

In the past two decades, studies have shown that some cytoplasmic proteins are surface associated in *H. pylori* e.g., urease, HSPA, HSP60, catalase, SOD (Hawtin *et al*., 1990; Phadnis *et al*., 1996; Mori *et al*., 1997; Vanet & Labigne, 1998). HSP20 is one of such proteins that has been identified in this study. As a surface associated cytoplasmic protein in *H. pylori,* it may indicate its functional importance for the bacterial pathogen against hostile gastric environment in host.

The mechanism responsible for the surface association of cytoplasmic proteins in *H. pylori* were explored. There are two possible ways to account for the release of cytoplasmic proteins: through either the way of bacterial autolysis or a specific secretion system. Both hypotheses are controversial among researchers. Under bacterial autolysis, it is claimed that many bacterial proteins appear in the supernatant of *H. pylori* broth culture and these proteins could attach onto the bacterial cell surface (Phadnis *et al*., 1996; Dunn *et al*., 1997). However, other researchers believed that some cytoplasmic proteins could be released into the extracellular space via specific or selective secretion system (Vanet & Labigne, 1998). The existence of both mechanisms in *H. pylori* is widely accepted in the studies of different proteins (Cao *et al*., 1998; Schraw *et al*., 1999).

Based on the results obtained from this study, the surface localized HSP20 could be released through a specific secretion system in *H. pylori*. It is because the extracellular attached proteins through bacterial autolysis would be removed from the cell surface of *H.*

pylori with the treatment of Triton X-100 before the step of immuno-labeling. However, HSP20 was still found on the cell surface or the remnants of cell surface after the treatment of Triton X-100 (Figure 4.9A & B). Hence, it implies that HSP20 is really associated with bacterial cell surface and this surface association is specific and tight. It is unlikely to be "sticking" onto the cell surface of *H. pylori*. Through specific secretion pathway, surface associated HSP20 could execute functions extracellularlly in *H. pylori*. This would further support the existence of specific secretion system in *H. pylori* for protein releasing specifically and selectively.

Surface localization of HSP20 may further address its unique function in *H. pylori* that is different from HslV of other bacteria. The observation of surface localized HSP20 like many other proteins (HSP60, urease, catalase, SOD) in *H. pylori* further highlights that cytoplasmic proteins becoming surface associated is a specific bacterial behavior which could be functionally important for *H. pylori* to combat against the hostile gastric environment (acidic & oxidative) in host.

5.3 Antibody against HSP20 in patients with gastroduodenal diseases

As a surface localized protein of *H. pylori*, the antibody against HSP20 in gastric patients' serum was analyzed using ELISA. However, the level of antibody against HSP20 in patients with various gastroduodenal diseases is comparable to that of the control subjects (Table 4.1). More importantly, the OD readings clustered within a very close range (0.21 ± 0.02). Among the same disease group, the antibody against HSP20 was not affected by the status of *H. pylori* infection (positive or negative). There are three possible reasons for this finding. Firstly, the similar level of antibody against HSP20

detected in the control subjects as that in the diseases group could be due to the cross reaction to its homologue (HslV) from other bacteria such as *E. coli* that is also present in gastrointestinal track. Secondly, low level of antibody against HSP20 detected could be due to an earlier exposure to *H. pylori* by both control subjects and patients alike, at a younger age. Since the association of *H. pylori* to humans was reported to have begun at an early age of 10 (Malaty *et al*., 2002) while the tested subjects in this study were all aged above 20, it might have resulted in a low but constant IgG level of HSP20. Finally, HSP20 may not be one of *H. pylori* major antigens inducing strong immune response during bacterial infection; thus there is no significant difference between patients and normal subject in the antibody level against HSP20.

It is noticeable that the ELISA readings ranged closely (0.21 ± 0.02) for all subjects detected. This is unlike the wide OD range between patients with gastro-duodenal diseases and control subjects when other antigens were used, such as CagA or VacA (Perez-Perez *et al*., 1999; Nomura *et al*., 2002). It implies that HSP20 might not be a suitable serological marker in detecting *H. pylori* infection. This may also indicate that HSP20 does not play a vital role in the initiation and induction of host immune response during *H. pylori* infection. This is unlike HSP60 that has been reported to be involved in chronic gastric inflammation following *H. pylori* infection in man (Yamaguchi *et al*., 2000).

However, as a surface localized protein, its role in adhesion similar to HSP60 cannot be overlooked. Like many other surface proteins of *H. pylori* playing important role in pathogenesis through adhesion, HSP20 may be involved in assisting colonization of the microorganism on the host. The importance of HSP20 in adhesion cannot be undermined,

as adhesion followed by colonization is a fundamental process in the course of *H. pylori* infection. Therefore, further study was carried out on surface localized HSP20 and its relation with the adhesion and colonization of *H. pylori.*

5.4 The role of HSP20 in *Helicobacter pylori*

The successful construction of *hsp20*-iosgenic *H. pylori* SS1 mutant has provided an opportunity to explore the potential role of HSP20 on the adhesion and colonization of *H. pylori in vitro* and *in vivo*.

5.4.1 HSP20 and adhesion of *H. pylori*

As a surface localized protein, the role of HSP20 in *H. pylori* adhesion was first examined under the *in vitro* conditions. In the *in vitro* adhesion analysis, insignificant difference was found in the adherence of *hsp20*-isogenic *H. pylori* as compared to that of the wild type, even though there was a reduction (8% - 26%) in the adhesion capability of the isogenic mutant. It indicates that *hsp20*-isogenic *H. pylori* retains almost similar adherence capability as that of the wild type. The slight decrease in adhesion capability of *hsp20*-isogenic *H. pylori* to AGS and Kato III cells under *in vitro* conditions implies that HSP20 is but one of the factors involved in the adhesion of *H. pylori* or that it may participate indirectly in the process of bacterial adherence. It therefore suggests and supports the role of various adhesins [e.g. BabA, OipA, HopZ, SabA and Le (X)] in *H. pylori* as reported by various studies on adhesion of *H. pylori* (Huesca *et al*., 1996; Yamaoka *et al*., 2000; Mahdavi *et al*., 2002; Odenbreit *et al*., 2002; Rad *et al*., 2002; Yamaoka *et al*., 2002). These multiple species of adhesins might function at different

stages of bacterial infection or cooperate in tandem. This is an area that needs further investigation.

5.4.2 HSP20 and colonization of *H. pylori*

The role of HSP20 in *H. pylori* colonization was further studied under *in vivo* conditions. Both wild type and *hsp20*-isogenic *H. pylori* were separately introduced into Balb/c mice by oral challenge to facilitate the study of HSP20 and bacterial colonization *in vivo*. The successful colonization of wild type *H. pylori* in mice stomachs was detected from 2 weeks of post-inoculation and persisted up to 8 weeks while there was no colonization detected in the mice inoculated with *hsp20*-isogenic *H. pylori* throughout the course of the 8-week study. The failure of *hsp20*-isogenic *H. pylori* to colonize in mice indicates that HSP20 protein may be essential for bacterial colonization.

Although there was no significant difference between wild type and *hsp20*-isogenic *H. pylori* on the adherence ability to gastric cell lines *in vitro*, *hsp20*-isogenic *H. pylori* lost the ability to colonize *in vivo* as compared to the wild type. This may indicate that the isogenic *H. pylori* could adhere to gastric mucus probably through the presence of multiple adhesins [e.g. OipA, HopZ, SabA and Le (X)] causing "transient colonization" for a short period of time but then loses its ability to colonize on the gastric epithelium of the animals (Dorrell *et al*., 1999). This result was also found in studying using other *H. pylori* mutants, e.g., the study of *flaA* mutant *H. pylori* in the gnotobiotic piglet model (Danon & Eaton, 1998), where aflagellated *H. pylori* mutant showed the similar adherence capability as that of the wild type *in vitro* but failed to colonize in the stomach of gnotobiotic piglet *in vivo*. Similar findings were also observed in the study of mutants

like *ureB H. pylori* mutant in gnotobiotic piglet model (Eaton & Krakowka, 1994) and *pldA H. pylori* mutant in mice model (Dorrell *et al*., 1999).

As proposed by researchers (Testerman *et al*., 2001), there are essentially two steps in the establishment of *H. pylori* in host: the initial adhesion followed by bacterial colonization. In the initial stage, many adhesins mediate the ligand-receptor interactions between bacteria and mucus layer of host to facilitate the "loose" attachment of *H. pylori* (Testerman *et al*., 2001). In the following stage, the bacteria begins to invade and colonize. In the process of colonization, bacterial pathogen may further damage the epithelium of host e.g., disruption of tight junction proteins (Amieva *et al*., 2003), induction of actin polymerization and cytoskeleton rearrangements of epithelial cells by means of releasing pathogenic factors (such as CagA) to assist further invasion (Testerman *et al*., 2001). Hence, that adhesion follows by colonization can be considered as two discrete but continuous processes, both of which are fundamental for commencement of bacterial infection. Therefore, compared with the adherence property, the role of HSP20 in bacterial colonization is more crucial since the impact of disrupted HSP20 is fatal in colonization of *H. pylori* in mice but not in bacterial adherence *in vitro*. It is therefore appropriate to imply that HSP20 is mainly responsible in assisting in the colonization of bacteria *in vivo*, apart from playing a minor role in adhesion.

5.4.3 Antibody response against *H. pylori* in mice model

As one of the major events occurred during the colonization of bacteria, total antibody response against the bacterial antigens, HSP20, was monitored using ELISA in both *H. pylori* infected animals. The importance of HSP20 in *H. pylori* colonization was

further highlighted by the presence of significantly lower antibody level against *H. pylori* in *hsp20*-isogenic mutant infected mice as compared with the mice infected with wild type bacteria. The low level of antibody response induced in *hsp20*-isogenic *H. pylori* infected mice (Figure 4.19A) can be related to the unsuccessful establishment of the bacterial pathogen on gastric epithelium of animals. This is probably because colonization is the prerequisite for the pathogenesis of *H. pylori*. Hence, this result further confirms the failure of *hsp20*-isogenic *H. pylori* in bacterial colonization.

5.4.4 Antibody against HSP20 in *H. pylori* infected mice

To examine the immunogenecity of surface localized HSP20 protein during *H. pylori* infection, specific antibody against HSP20 in the sera of *H. pylori* infected mice was examined using ELISA. The results showed that there is no significant difference in the antibody against HSP20 in both wild type and *hsp20*-isogenic *H. pylori* infected mice (Figure 4.19D). Furthermore, the level of antibody against HSP20 in *H. pylori* infected mice was within the same range as that of the negative controls. This may indicate that the absence of HSP20 in the isogenic *H. pylori* is not directly correlated with the low antibody production in the mutant infected mice. It could further attest that HSP20 is not a major antigen inducing strong and constant immune response during *H. pylori* infection. This implication agrees with the previous finding obtained in the detection of antibody against HSP20 in gastroduodenal diseases patients (Table 4.1). Hence, this also implies that antibody production in *H. pylori* infected mice would be related to the other bacterial surface antigens but not HSP20.

5.5 Protein profiles of wild type and *hsp20*-isogenic *H. pylori*

With the development of proteomic technologies, the proteome, a functional part of the genetic information provides new perspectives to reveal the association of protein product with the pathogenesis of *H. pylori* (e.g. to identify new virulent or pathogenic factors) (McAtee *et al*., 1998). Comparative proteome analysis of *H. pylori* showed that the bacterial proteome varied from strains to strains (Jungblut *et al*., 2000) and this might be related to the virulence of the stains. It is therefore useful to examine whether the proteome of *hsp20*-isogenic *H. pylori* is different from the wild type or whether HSP20 could affect the expression of proteins related to the bacterial colonization. This could help us to understand the reason why *hsp20*-isogenic *H. pylori* failed to colonize in mice.

The protein profiles of both wild type and *hsp20*-isogenic *H. pylori* were analyzed using SDS-PAGE and two-dimensional gel electrophoresis (2-DE). The comparison of protein profiles showed that no observable difference was found between the wild type and the isogenic *H. pylori* (Figure 4.20). This may indicate that the disrupted HSP20 would not influence the expression of major proteins in *H. pylori* although there is limitation in resolving the entire protein expression profiles.

5.6 Gene status of *Helicobacter pylori* adhesins

A number of adhesins in *H. pylori* have been revealed in recent years such as BabA (Boren *et al*., 1993; Ilver *et al*., 1998), OipA (Yamaoka *et al*., 2000), HopZ (Peck *et al*., 1999) and SabA (Mahdavi *et al*., 2002). It has been found that under *in vitro* or *in vivo* (in mice) passages, the status of the adhesin genes in *H. pylori* could be switched from “on”

(in-frame) to "off" (out-of-frame) that would affect the adhesion and colonization of *H. pylori* in mice (Yamaoka *et al*., 2000).

Based on the DNA sequence analysis of CT repeats in the signal sequence of adhesin genes, it was found that *oipA*, *hopZ* and *sabA* genes were switched "on" in both wild type and *hsp20*-isogenic *H. pylori* (Table 4.4). However, the other adhesin gene, *babA2* was shown to be absent in *H. pylori* SS1 strain. The "on" status of the three major adhesins was further confirmed by RT-PCR analysis where the adhesin genes were transcribed in both wild type and *hsp20*-isogenic *H. pylori*. This finding suggests that the failure of *hsp20*-isogenic *H. pylori* colonizing in mice is not related to the switching status of the various adhesin genes of *H. pylori* studied. It is also suggested that HSP20 might not directly interact with these molecules. Furthermore, it may imply that the effect of HSP20 on the colonization of *H. pylori* is independent of the major adhesins studied.

5.7 Protein interaction between HSP20 and CagA in *Helicobacter pylori*

In an attempt to study the interaction between HSP20 and other proteins in *H. pylori*, co-immunoprecipitation (CO-IP) using antibody against rHSP20 showed that a single protein CagA was pulled down from the protein extract of the wild type but not the isogenic *H. pylori* (Figure 4.22 & 4.24). This indicates that HSP20 would potentially interact with CagA in *H. pylori*. The absence of this interaction in *hsp20*-isogenic *H. pylori* and other bacteria (*E. coli* and CagA-negative *H. pylori*) serving as the negative controls confirms that the interaction between HSP20 and CagA is novel and unique.

Although *cag* PAI was reported not to be functional in *H. pylori* SS1 with lack of ORF 7 (cag^{+}ORF7^{-}) (Salama *et al*., 2000), the recent mutagenesis study by Fischer *et al*.,

(2001) showed that ORF 7 (HP0521) has no effect on either the translocation of CagA from *H. pylori* to gastric cells or the ability of *cag* PAI to induce IL-8 production of host during *H. pylori* infection. In addition, there was no other component from *cag* PAI precipitated down together with CagA protein by using antibody against rHSP20. Thus, it is appropriate to believe that HSP20 only interacts with CagA protein but no other component of *cag* PAI. Thus, these findings indicate that the interaction between HSP20 and CagA is independent of *cag* PAI but relies on the functional CagA protein alone.

However, how HSP20 collaborates with CagA is yet to be determined. Since the interaction between HSP20 and CagA was absent in the *hsp20*-isogenic *H. pylori*, whether HSP20 affects the expression of CagA and further related to the presence of CagA in different sub-cellular fractions were analyzed using RT-PCR and Western blotting.

5.7.1 RT-PCR analysis of *cagA* transcription

Based on the RT-PCR analysis of *cagA* in both *H. pylori* strains, it was found that the transcription level of *cagA* in the isogenic *H. pylori* is similar as that of the wild type regardless of different ages of *H. pylori* culture examined (Figure 4.25). This indicates that the transcription of *cagA* gene remains normal and would not be affected by the disrupted HSP20 in the isogenic mutant. It may further imply that the influence of HSP20 on CagA in *H. pylori* is not at genetic level but at protein level.

5.7.2 Analysis of CagA protein partition in *H. pylori*

As a major virulence factor of *H. pylori* (McGee & Mobley, 1999), CagA was firstly identified as a surface antigen of *H. pylori* that is associated with the cytotoxin production (Tummuru *et al*., 1993). The surface exposed CagA in *H. pylori* is not only related to the induction of antibody in host but also linked with duodenal diseases (Covacci *et al*., 1993; Crabtree & Lindley, 1994). Hence, the presence of CagA on the cell surface of *H. pylori* is very important for bacterial virulence. Although CagA has been found to be translocated from bacterial cytoplasm into the host epithelial cells through type IV secretion system during *H. pylori* infection (Segal *et al*., 1999; Stein *et al*., 2000; Backert *et al*., 2000), the process of CagA becoming surface exposed as an antigen has not been studied. Therefore, the relevance of HSP20 to the presence of CagA protein in different *H. pylori* sub-cellular fractions was investigated.

With the help of WB, it was shown that the presence of CagA in different sub-cellular fractions was slightly different between the wild type and *hsp20*-isogenic *H. pylori*. The absence of CagA in AGE fraction (acid glycine extract comprising membrane and membrane associated proteins) and a comparatively lower abundance of CagA protein in OMP fraction (outer membrane proteins) of the isogenic *H. pylori* (Figure 4.26A) could be correlated to the disruption of HSP20. Interestingly, with the addition of rHSP20 into the isogenic *H. pylori* culture, CagA emerged in all these 3 fractions [total proteins (TP), AGE & OMP] of the isogenic mutant with similar intensity as that of the wild type. (Figure 4.26B). This further strengthens the relationship between HSP20 and the presence of CagA in *H. pylori*.

Since OMP and AGE fractions which comprises membrane and membrane associated proteins represent the surface part of *H. pylori* (Goodwin *et al*., 1987) and the necessity of surface presentation of CagA to bacterial pathogenensis (Covacci *et al*., 1993), the close relationship between HSP20 and the presence of CagA in the surface fractions (AGE and OMP) highlights that HSP20 is important in collaborating with the presentation of CagA in *H. pylori*. It is postulated that the disrupted HSP20 in the isogenic *H. pylori* had resulted in the decreased amount of CagA in OMP fraction but was compensated by the addition of rHSP20 during culturing. Hence, it would further support that HSP20 is relevant to the presence of CagA protein in different *H. pylori* sub-cellular fractions especially OMP and AGE fractions. Furthermore, the absence of CagA in the AGE fraction of the isogenic mutant while its restoration with the addition of rHSP20 may imply that HSP20 could be required for the stabilization of CagA under low pH conditions, similar to the acidic environment in the stomach of the host (as AGE was prepared at pH 2.2).

5.7.3 Antibody against CagA in *H. pylori* infected mice

As a major antigen in *H. pylori*, CagA could induce high antibody titer in humans and the infected animals during bacterial infection (Wirth *et al*., 1998; Loffeld *et al*., 2000; Dzierzanowska-Fangrat *et al*., 2003). Hence, the potential collaboration between HSP20 and the presentation of CagA on *H. pylori* was further tested using antibody against CagA in *H. pylori* infected mice. The antibody level against CagA in *hsp20*-isogenic *H. pylori* infected mice was found to be significantly lower ($p<0.05$) than that of the wild type (Figure 4.27). This may explain the good correlation between low abundance of

CagA detected in the surface protein fraction of the isogenic *H. pylori* and the low antibody response against CagA in the isogenic mutant infected mice. It is possible that the disrupted HSP20 in the isogenic mutant could have led to the ineffective presentation of CagA or partial CagA as antigen on the cell surface of *H. pylori* resulting in the reduction of antibody induced during *H. pylori* infection. This may further support the probable involvement of HSP20 in the presentation and stabilization of CagA on the cell surface of *H. pylori*.

Based on the findings obtained from the relation between HSP20 and CagA in *H. pylori*, it is clear that disrupted HSP20 does not affect the transcription of *cagA* in *H. pylori* but influence the presence of CagA protein in different bacterial sub-cellular fractions. The correlation of HSP20 with the presentation of CagA on the cell surface of *H. pylori* may imply that HSP20 could be involved in such process of CagA presentation. As a heat shock protein, HSP20 could probably function as an assisting factor in "delivering" and/or "presenting" CagA onto the cell surface of *H. pylori*. It is therefore proposed that HSP20 may serve as a "chaperon" for the virulence factor, CagA in *H. pylori*.

On the other hand, owing to the significant contribution of CagA to the pathogenecity of *H. pylori* infection, the indirect association of HSP20 with the bacterial pathogenesis through CagA is further highlighted. From the identification of interaction between HSP20 and CagA to the possible chaperonic role of HSP20 for surface presentation of CagA, it further addresses the importance of heat shock protein in bacterial infection and the unique function of HSP20 in *H. pylori* which differs from that of the HslV in *E. coli*.

5.8 The application of HSP20 as an epidemiological and gastroduodenal disease differentiating marker

As a surface localized protein, HSP20 has shown strong association with bacterial colonization and indirect relationship to the pathogenesis of *H. pylori*. It plays a cooperative role in the initial stage of *H. pylori* infection implying its importance in the bacteria-host interactions. Due to the significant contribution of HSP20 in the process of *H. pylori* infection, it is therefore interesting to understand the genetic structure of HSP20 in *H. pylori* population. The relevance of *hsp20* gene to differentiate various *H. pylori* infections and for use as a marker for epidemiology of *H. pylori* was explored.

5.8.1 Conservation and polymorphism of *hsp20* DNA sequence

The DNA sequences of 227 *H. pylori* strains collected from different parts of the world showed that *hsp20* (HP0515) gene is highly conserved. It showed consistent and stable open reading frame (ORF) of 540 bp in all *H. pylori* isolates studied. This is different from *cagA*, *vacA* and outer membrane protein (HP0638) gene sequences where gene polymorphism has led to insertion or deletion of gene fragments among different *H. pylori* strains. For instance, *vacA* has at least two variable regions: s region (s1a, s1b, s1c & s2) and m region (m1 & m2) (van Doorn *et al*., 1999); similarly, HP0638 shows two dichotomies that is strongly correlated with *cagA* and *vacA* status (Ando *et al*., 2002) while *cagA* gene is only present in 60% - 70% of *H. pylori* strains (Mobley, 1997) among the Western population but was found in 80% - 90% of *H. pylori* isolates in Asian population (Zheng *et al*., 2000). The conservation of *hsp20* gene in all *H. pylori* strains

indicates that intact gene structure of heat shock proteins is essential for implementation of protein functions.

Among the 227 DNA sequences of *hsp20* gene studied, high level of polymorphism at 219 polymorphic sites scattered in the whole gene fragment. The occurrence of polymorphism within the same gene fragment in different organisms or strains would take place spontaneously during the evolution and extensively observed in nature. The presence of high polymorphism in *hsp20* gene signifies that identical DNA sequences will be rare among *H. pylori* strains. This lends support to the existence of high level of genetic diversity among *H. pylori* isolates. This finding agrees with several previous reports in which the studies were based on the analyses of other gene fragments of *H. pylori* (Ito *et al*., 1996; Salaun *et al*., 1998; Janssen *et al*., 2001).

5.8.2 Geographical groupings of *H. pylori* phylogeny based on *hsp20* DNA sequences

Based on the phylogenetic analysis of *hsp20* DNA sequences, two major clusters (A & B) were shown: a larger group A and a smaller group B (Figure 4.28). The isolates in group A further bifurcates into two subgroups, namely A_1 and A_2. Subgroup A_1 and group B were mainly from Asian and non-Asian origins, respectively. However, subgroup A_2 comprises a mix of *H. pylori* isolates of Asian and non-Asian origins. From the geographical character and the percentage of geographical distribution of *H. pylori* isolates in these two groupings (A & B), it shows that there is a pattern of transitional clustering from predominate Asian (group A_1) to a mixture of Asian & non-Asian origins (group A_2) followed by a majority of *H. pylori* isolates of non-Asian origin (group B).

This pattern points out the possible long-term influence of worldwide population migration and human activities on the infection and/or transmission of *H. pylori*. It also implies that the evolution of Asian and non-Asian *H. pylori* strains was not totally independent from geographical separations. For example, four Singapore isolates of Malay ethnic origins were clustered within group B and a number of non-Asian isolates were clustered with group A. This could be due to the closeness of geographical regions e.g., Australia is close to South-east Asia; or the similar host response to the pathogen e.g. Malay and European as reported by (Goh, 1997). The similarity between Malay and European could be either in the genetic susceptibility or in the immune response to *H. pylori* infection. The results on the geographical groupings agree with a number of previous studies in which the analyses were based on the sequence analyses of house-keeping genes e.g. *recA*, *atpD*, *glnA*, *scoB* or genotyping of virulence genes *cagA*, *vacA* and transposable elements IS605 and IS608 (Salaun *et al*., 1998; Maggi *et al*., 2001; Ji *et al*., 2002; Kersulyte *et al*., 2002).

According to the inferred taxonomic distance calculated, it implies that group B (non-Asian origin) would have diverged from group A (predominately Asian origin, as shown in Figure 4.28). This is depicted by the clusters in the phylogram and the estimated nucleotide divergence (*D*) between different groups. Since there is no evidence that synonymous codon usage is constrained in *H. pylori*, *Ks* should roughly reflect the divergence time between sequences (Atherton *et al.*, 1999). Based on the order of estimated value of *Ks* between groups (Table 4.5) and as shown in the phylogram (Figure 4.28), it indicates that the division of A_1/B was the earliest followed by the divisions of A_2/B and A_1/A_2. Furthermore, the significant lower divergence differences between

hsp20 gene groups (Table 4.5) than that of the *vacA* alleles imply that the clusters based on *hsp20* gene are only modestly subdivided geographically as are the housekeeping genes claimed by Achtman *et al*. (1999).

Compared with other genes of *H. pylori*, the percentage of divergence (*D, Ks*) and the ratio of *Ks/Ka* between different *hsp20* gene groupings were significantly lower than that of *vacA* alleles m1 & m2 (Atherton *et al*., 1999) or house-keeping genes (*atpD*, *scoB*, *glnA* and *recA*) (Maggi *et al*., 2001) as reported. This may indicate that different genes of *H. pylori* are under the control of different selection pressures against amino acid replacement (Hurst, 2002). The divergence ((*D, Ks, Ka*) and the ratio of *Ks/Ka* values (Table 4.5) in the 3 different genetic groupings show that *hsp20* sequences are stable and effective in discriminatively distinguishing *H. pylori* strains from different geographical origins. The evolutionary variations of *H. pylori* based on the findings of *hsp20* phylogeny are comparable with that of housekeeping genes and surface proteins (HP0638). As the proteins of initiating interaction between bacteria and host cells, surface proteins would be responsible for various responses to the extracellular environments and may be involved in the bacterial-host interactions during the process of *H. pylori* infection.

5.8.3 Substitutions at 14^{th} – 16^{th} amino acid residues of HSP20

There were seven types of substitutions observed at the 14^{th} –16^{th} amino acid residues. Interestingly, the switching of substituted amino acids were within the same polarity group e.g. from hydrophobic to hydrophobic (e.g., 14^{th}: M, I, L & F) or from polar to polar (e.g., 15^{th}: G, R, E, N & D; 16^{th}: G, N & H) resulting in no modification in protein

conformation as supported by the predicted secondary and 3-D structure. Thus, the amino acid substitutions in HSP20 have no impact on the protein structure (Figure 4.30 & 4.31), indicating that HSP20 protein structure is not random but well conserved. The conservation of HSP20 protein structure is in accordance with its gene structure, which further strengthens it as one of the constitutively expressed proteins in microorganisms. Furthermore, like other heat shock proteins, maintaining the stability and consistency is essential for normal function of most known heat shock proteins (Ang *et al*., 1991; Jaattela & Wissing, 1992).

Although HSP20 (HP0515) is a homologue of HslV in *Haemophilus influenzae* (Sousa *et al*., 2002) and *E. coli* (Song *et al*., 2000), the 3-amino-acid substitutions at 14^{th} $–16^{th}$ is unique in HSP20 as it is not present in the homologues of these two bacterial species (Figure 4.32), implying that these 3-amino-acid residues could have acquired during the evolutionary process of *H. pylori* and may represent the adaptation of bacteria to different environments or reflect the random genetic drift without effects on phenotype. Hence, it further indicates the distinctiveness of *hsp20* and the 3-amino-acid substitutions.

It is interesting to note that the unique 3-amino-acid substitutions of all HSP20 amino acid sequences exhibited similar geographical affiliation as the *hsp20* DNA sequences. In the former, these two clusters can be divided into two broad groups: M-G-G and F-D-N clusters. The change of substitutions occurs with the transition of one or more nucleotide, e.g. in the case of M-G-G and I-G-G, the substitution from methionine (M) to isoleucine (I) would have resulted from the nucleotide transition of ATG [methionine (M)] to ATA or ATT [isoleucine (I)]; similarly, the other substitutions M-G-G to M-E-G and M-R-G occurred with a transition of one nucleotide for the middle glycine G, (GGG) to

glutamate E, (GAG) or arginine R, (AGG) (Figure 5.1). This is considered as M-G-G substitution cluster. The other substitution cluster comprises F-D-N, F-N-H & L-N-H that is termed as F-D-N cluster. Unlike the M-G-G cluster which nucleotide substitution occurs in one single step, the F-D-N linkage cluster needed more than single "mutation" from the original to L-N-H & F-N-H (Figure 5.1). It is apparent that the M-G-G substitution cluster in group A and F-D-N substitution cluster in group B are completely separated from each other, which are strongly associated with *H. pylori* isolates of Asian and non-Asian origins respectively.

Based on the process of nucleotide substitutions as illustrated in Figure 5.1 and the contribution of Asian M-G-G linkage type (82%, 143/172) in all isolates studied, it is postulated that *H. pylori* with M-G-G linkage has its origin in Asia. The origin of M-G-G from Asian is explicit in its prevalence and the simple process of nucleotide substitutions. Among the observed substitution linkages, M-G-G forms a big base in both Asian (143/158, 90%) and non-Asian groups (29/69, 43%); however, the majority of M-G-G were from Asia. Both Asian and non-Asian originated M-G-G types were evidently similar to each other especially in group A_2. In the proposed process of nucleotide transitions in all substitution linkages, it is likely that the transition could occur from M-G-G to the other types since the chemical structure of G and M are the simplest and most stable among all substituted amino acids observed (Branden & Tooze, 1998). It may also imply that there could be 2 precursors (M-G-G and F-D-N linkages) that have come from one single universal ancestor. However, this does not exclude the postulation that *H. pylori* begins in Asia with M-G-G linkage. It is not only because that M is the most stable residue among the all substituted amino acids; but also *H. pylori* infection is more

prevalent in Asia and the most problematic cases of gastroduodenal disease (e.g. gastric cancer) are in Asia. Hence, it further supports the speculation that M-G-G originated from Asian.

Besides the affiliation of the two major substitution clusters in their discriminatory capability based on geographical origins (with prevalence of M-G-G cluster in Asian and F-D-N cluster in non-Asian areas), these two substitution clusters also showed significant association with clinical outcome which is supported by the calculated odds ratio (OR = 4.27) (Table 4.7). Of these, M-G-G cluster is shown to be associated with PUD as compared to the association of F-D-N cluster to NUD. The link of M-G-G among Asians with PUD and F-D-N in non-Asians with NUD may further highlight that *H. pylori* together with environmental factors are collaboratively contributing to the gastroduodenal disease outcome. The results thus show the association of M-G-G cluster in the Asian group where the PUD is more prevalent (Kang *et al*., 1997; Lam, 2000). However, the results do not exclude the presence of PUD outside this geographical region as there is the existence of other collaborative factors that can contribute to the pathogenesis of *H. pylori* and severity of gastroduodenal diseases. Rather, this study emphasizes the usefulness of the substitution clusters (14^{th} – 16^{th}) of HSP20 as an indicator to evaluate the risk of developing certain gastroduodenal diseases (PUD or NUD).

The conserved *hsp20* DNA sequences in all *H. pylori* strains studied have presented a foundation for its use as epidemiological marker. With these DNA sequences, two phylogenetic groups based on Asian and non-Asian origins were effectively differentiated. This differentiating capability is also displayed by the unique 3-amino-acid substitutions

at 14th – 16th residues of HSP20. Furthermore, the 3-amino-acid substitutions clusters (M-G-G and F-D-N clusters) show significant discriminating efficiency between PUD and NUD. We therefore propose *hsp20* and the novel insertion of 3-amino-acid substitution clusters as a potential epidemiological and gastroduodenal disease differentiating marker of *H. pylori* infection.

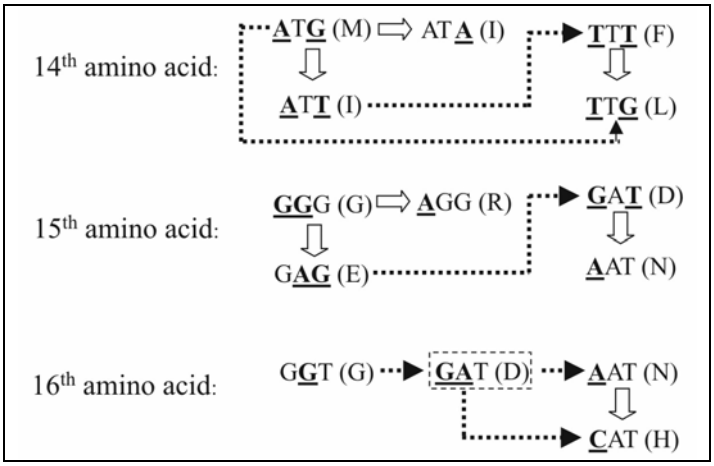


Figure 5.1 The probable process of nucleotide substitution sequence in 14th – 16th amino acid residues

Letters in parentheses denote amino acids. Substituted nucleotides are bolded and underlined. Arrows indicate probable process of nucleotide substitutions.

The probable substitutions are based on the sequences generated in this study, e.g. at 15th amino acid, for glycine (G), only GGG/GGC were recorded while for 16th amino acid, all three DNA substitutions GGT, GGC and GGG were observed.

The figure is merely a proposed process of probable nucleotide substitution.

5.9 Conclusion

From the identification of surface localization of HSP20, the low titer antibody against HSP20 detected in patients' sera to the involvement in the bacterial colonization and the interaction with virulence factor CagA, HSP20 manifests its importance and uniqueness in *H. pylori*. As a human gastric pathogen, the chaperonic function of HSP20 in *H. pylori* is primarily rendered by its unique surface localization, which is different from the cytoplasmic located HslV of *E. coli*. Since its unique location on *H. pylori* cell surface, the role of HSP20 and host immune response in *H. pylori* infection was studied. With the presence of low antibody level against HSP20 as detected in various gastroduodenal patients, it implies that HSP20 might not be one of the major antigens provoking strong immune response of host during *H. pylori* infection. However, as a surface localized protein, its role in assisting the colonization of *H. pylori* is highlighted.

In light of its potential in bacterial colonization, comparison studies between wild type and *hsp20*-isogenic *H. pylori* demonstrated that HSP20 would be involved in the colonization of *H. pylori*, which is an essential step for the establishment of bacterial pathogen and the development of pathogenesis during infection. HSP20 is indispensable for bacterial colonization but not necessary in the adhesion of *H. pylori*. Low-level antibody against *H. pylori* total antigens in the mice infected with *hsp20*-isogenic *H. pylori* further verifies the cooperative role of HSP20 in bacterial colonization. Moreover, the role of HSP20 on the bacterial colonization is independent of the major adhesins in *H. pylori*. The findings observed in this study lend support to the fact that the sequential events occurred during *H. pylori* infection start from adhesion to colonization and eventually to inducing the immune responses of the host. It is noted that these events

occur progressively coherent to each other. Disabling any one of the components could result in disastrous consequences in the pathogenic process of *H. pylori*. This correlation have been extensively observed in the study of other different surface protein molecules e.g., adhesins (Boren *et al*., 1993; Ilver *et al*., 1998), urease (Tsuda *et al*., 1994; Karita *et al*., 1995), GGT (Chevalier *et al*., 1999; McGovern *et al*., 2001) or phospholipase (Dorrell *et al*., 1999) and outer membrane proteins (Yamaoka *et al*., 2002), which have been experimentally shown to be correlated with the adhesion and colonization of *H. pylori*. It also addresses the significance and importance of bacteria-host interplay during the infection.

The elucidation of interaction between HSP20 and CagA further reveals a possible link between HSP20 and the pathogenesis of *H. pylori* through CagA. In the analysis of relationship between HSP20 and CagA, the cooperative role of HSP20 acting like a "chaperon" in assisting the "presentation" of CagA on the cell surface of *H. pylori* was noted. As a heat shock protein, HSP20 may also be required for the stabilization of CagA in *H. pylori*. Such uniqueness signifies that the effective CagA protein of *H. pylori* requires the aid of other assisting factors such as heat shock protein to serve as chaperon so as to fulfill its function during bacterial infection. As demonstrated by many other researchers (Crabtree, 1996; Yamaoka *et al*., 1996; Eck *et al*., 1997; Graham & Yamaoka, 1998), CagA is an important virulence factor directly related to the pathogenesis of *H. pylori*. Thus, the chaperonic role of HSP20 in the pathogenesis of *H. pylori* is further highlighted indirectly through its link with CagA. On the other hand, the chaperonic role of HSP20 in *H. pylori* may further imply that the acquired novel function of heat shock proteins (e.g., HSP20) is evolutionarily adapted to the virulence of bacterial pathogen,

which differs from HslV (the homologue of HSP20) of other bacteria, thereby indicating the sophistication and uniqueness of *H. pylori*.

As a surface localized protein and significant contribution to the pathogenesis of *H. pylori*, use of HSP20 in the epidemiological study of *H. pylori* was explored. Based on the analysis of nucleotide sequences of *hsp20* from 227 different *H. pylori* strains, it indicates that *hsp20* is dependent from other genes (*cagA, vacA,* transposable elements, some housekeeping genes) in the delineation of *H. pylori* isolates worldwide. The geographic grouping of *hsp20* also exhibits the affiliation with the distinctive 3-amino-acid substituions at 14^{th} – 16^{th}. The bifurcating of M-G-G type and F-D-N type are not only coupled with Asian and non-Asian groupings and also strongly associated with PUD and NUD, respectively. That may provide a simple but effective approach for researchers to locate the possible source or route of *H. pylori* infection and differentiate its associated gastroduodenal diseases (PUD & NUD).

With the identification of HSP20 being essential for *H. pylori* colonization and interaction with CagA, it opens a new insight to elucidate bacterial-host interaction in the progress of *H. pylori* infection. Animal model of *hsp20*-isogenic *H. pylori* provides an unprecedented negative control system for bacterial infection to facilitate other studies of *H. pylori in vivo*.

5.10 Future work

Recent study by Amieva *et al*. (2003) had shown that CagA could disrupt the tight junction protein complex of host cells at the site of the bacterial attachment during *H. pylori* infection. Disruption of the tight junction protein complex can be considered as an

early event occurred in the colonization of *H. pylori*. Hence, studying the protein-protein interaction between HSP20 and CagA could provide useful information in understanding the indirect relationship between HSP20 and tight junction proteins (eg ZO-1, occludin). The investigation on HSP20-CagA-tight junction complex/proteins may shed light into the intimate links between protein structure and their function(s) in the host-pathogen relationship and lend an insight into the mechanism of pathogenesis of *H. pylori* infection.

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7. APPENDIX

1. 6X Agarose gel loading buffer

Bromophenol blue (0.25%)	0.25 g
Xylene cyanol (0.25%)	0.25 g
EDTA (50 mM)	18.76 g
Glycerol (30%) [v/v]	30 ml
Distilled water	100 ml qsp

2. Brain-heart Infusion broth (BHI)

Brain-heart infusion medium	3.8 g
Yeast extract (0.4%)	0.4 g
Distilled water	90 ml qsp

Autoclave at 121 ºC for 15 minutes. Add 10 ml (10%) horse serum before use.

3. Buffers for affinity chromatography

Binding buffer

5 mM imidazole	0.034 g
0.5 M NaCl	2.92 g
1 M Tris-Cl (pH 7.9)	2 ml
Distilled water	100 ml qsp
Adjust pH to 7.9	

Charge buffer

50 mM $NiSO_4$	0.78 g
Distilled water	100 ml qsp

Elution buffer

1 M imidazole	6.8 g
0.5 M NaCl	2.9 g
1 M Tris-Cl (pH 7.9)	2 ml
Distilled water	100 ml qsp
Adjust pH to 7.9	

Stripping buffer

0.5 M EDTA (pH 8.0)	20 ml
0.5 M NaCl	2.92 g
1 M Tris-Cl (pH 7.9)	2 ml
Distilled water	100 ml qsp

Wash buffer

60 mM imidazole	0.41 g
0.5 M NaCl	2.92 g
1 M Tris-Cl (pH 7.9)	2 ml
Distilled water	100 ml qsp
Adjust pH to 7.9	

4. Buffers for plasmid extraction

Solution I (for plasmid extraction)

50 mM glucose	0.9 g
1 M Tris–Cl (pH 8.0)	2.5 ml
0.5 M EDTA (pH 8.0)	2 ml
Distilled water	100 ml qsp

Autoclave at 115 ºC for 15 minutes and store at 4 ºC until use.

Solution II

0.2 M NaOH	20 ml
1% SDS	10 ml
Distilled water	100 ml qsp

Prepare before use.

Solution III (plasmid extraction)

5 M potassium acetate	60 ml
Glacial acetic acid	11.5 ml
Distilled water	28.5 ml

5. Chocolate blood agar

Blood agar base	20 g
Distilled water	475 ml qsp

Autoclave at 121 ºC for 15 minutes. Cool medium to 50 ºC and add 25 ml (5%) horse blood. Heat medium at 80 ºC for 10 minutes to lyse blood before pouring.

6. Coating buffer (for ELISA) – [0.1 M Carbonate buffer]

$NaHCO_3$	8.4 g
Na_2CO_3	3.56 g
Distilled water	1000 ml qsp

Adjust pH to 9.5

7. 0.5 M EDTA pH 8.0

EDTA	146.1 g
Distilled water	1000 ml qsp

Adjust pH to 8.0

8. Luria Broth (LB) medium

NaCl	10 g
Yeast extract	5 g
Tryptone	10 g
Distilled water	1000 ml qsp

Adjust pH to 7.2 to 7.4. Autoclaved at 121 ºC for 15 minutes.

9. LB agar

NaCl	10 g
Yeast extract	5 g
Tryptone	10 g
Agar	20 g
Distilled water	1000 ml qsp

Adjust pH to 7.2 - 7.4. Autoclave at 121 ºC for 15 minutes.

10. 1 M NaOH

NaOH	
Distilled water	1000 ml

11. Phosphat buffer saline (PBS) pH 7.4

NaCl	8.0 g
Na_2HPO_4	2.9 g
KCl	0.2 g
KH_2PO_4	0.2 g
Distilled water	1000 ml qsp
Adjust pH to 7.4.	

12. PBS-T buffer (pH 7.4)

NaCl	8.0 g
Na_2HPO_4	2.9 g
KCl	0.2 g
KH_2PO_4	0.2 g
Tween 20 (0.5%)	0.5 ml
Distilled water	1000 ml qsp

13. 10% SDS (W/V)

SDS powder	10 g
Distilled water	100 ml qsp

14. Substrate buffer (for ELISA)

Citric acid	2.5527 g
Na_2HPO_4	4.5746 g
Distilled water	500 ml qsp

15. SDS-PAGE gel preparation

	10%	12%	15%	5%
*30% acrylamide	1.7 ml	2 ml	2.5 ml	0.83 ml
1.5 M Tris-HCl (pH 8.8)	1.3 ml	1.3 ml	1.3ml	0.63 ml (1 M Tris-HCl pH6.8)
10% APS	50 µl	50 µl	50 µl	50 µl
10% SDS	50 µl	50 µl	50 µl	50 µl
TEMED	2 µl	2 µl	2 µl	5 µl
DD H_2O	1.9 ml	1.6 ml	1.1 ml	3.4 ml
Total volume	5 ml	5 ml	5 ml	5 ml

- acrylamide stock solution was purchased from Bio-rad. (adapted from (Bollag *et al.*, 1996)

16. TE buffer (pH 8.0)

1 M Tris-Cl (pH 8.0)	10 ml
0.5 M EDTA (pH 8.0)	2 ml
Distilled water	1000 ml qsp

17. 5 X Tris-acetate EDTA (TAE) buffer

Tris base (40 mM)	24.2 g
Glacial acetic acid	5.71 ml
EDTA (2 mM)	3.72 g
Distilled water	1000 ml qsp

18. 1 M Tris-Cl pH 7.9

Tris base	121 g
Distilled water	1000 ml qsp
Adjust pH to 7.9	

19. 1 M Tris-Cl pH 8.0

Tris base	121 g
Distilled water	1000 ml qsp
Adjust pH to 8.0	

8. PUBLICATIONS

Surface localized Heat Shock Protein 20 (HslV) of *Helicobacter pylori*

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ABSTRACT

Background. Heat Shock Protein (HSP) has been regarded as a pathogenic factor in *Helicobacter pylori* infection. Heat Shock Protein 20 (HSP20) of *H. pylori* is identified as Hs1V based on open reading frame predication of genome sequences. It is a homologue of HslV of *E. coli*, a peptidase involved in protein degradation.
Methods. The HSP20 gene was cloned and inserted into pET16b fused with His-tag. Recombinant HSP20 protein (rHSP20) was expressed and purified by nickel column. Rabbit anti-rHSP20 was purified by Protein A affinity chromatography and used as a probe to localize HSP20 in *H. pylori* by immuno-gold labeling and Western blotting. rHSP20 was also used as antigen to test for antibody against HSP20 in patients with *H. pylori* infection by enzyme-linked immunosorbant assay.
Results. Immuno-gold labeled transmission electron microscopy shows that HSP20 is located on the cell surface of *H. pylori*. Western blotting of 2-D gel shows that HSP20 has a pI of ~5.5 and a molecular weight of ~18 kDa. The ELISA result shows that there is no significant difference in antibody titre against rHSP20 in all sera tested.
Conclusion. The presence of IgG to rHSP20 may imply an earlier exposure of the patients and normal subjects to *H. pylori*. However, the mechanism has not been established. HSP20 has been shown to localize on the surface of *H. pylori*. Surface localization of *H. pylori* HSP20 may provide the path to a better understanding of the role and function of HSP20 in bacteria–host interaction.

Helicobacter pylori is a gram negative, spiral-shaped bacterium that colonizes human gastric stomach. It is associated with different gastro-duodenal diseases ranging from gastritis to gastric carcinoma [1]. However, the mechanisms of *H. pylori* infection, transmission and associated diseases have not been established [2]. Heat Shock Protein (HSP) has been considered as a virulence factor of bacteria that helps in stabilizing proteins and bacterial survival under stress [2]. Among the many HSPs identified in *H. pylori*, HSP60 homologue shares ~50% similarity with human HSP60 [3]. It plays an important role in stimulating adhesion, triggering autoimmune response, and inducing cytokine production in *H. pylori* infections [4,5].

HSPs are a group of highly conserved proteins widely expressed in prokaryotes and eukaryotes. Under normal conditions, expression of HSP is maintained at basal level serving as a molecular chaperon in protein folding and degradation. A variety of factors could induce the heat shock response and regulation of HSP expression, such as analogues of amino acid, growth factors, virus infection and stress [6].

Recently reported HslVU is a small HSP expressed in *E. coli* under the *hslVU* operon, which is involved in protein degradation [7]. It is widely believed that HslVU is an ATP-dependent protease including a 19KDa HslV and a 50KDa HslU [8]. HslV harbours the peptidase activity while HslU itself provides an essential ATPase activity; together they function as a two-component protease to degrade SulA, a cell division inhibitor in *E. coli* [7]. It has also been shown that the proteolytic activity of HslV can be dramatically increased by the presence of HslU [9]. The primary structure of HslV is similar to that of human β-type subunits of 20S proteosomes [9]. A series of HslV homologues has been reported in different bacteria such as *Bacillus subtilis* [8], *Leptospira borgpetersenii* [10] and *Lactobacillus leichmannii* [11].

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Homologues to HslV and HslU were predicted in the genomic sequence of *H. pylroi* strain 26695 [12]. In this study, an HslV homologue in *H. pylori*, referred to as HSP20, shares ~57% identity with *E. coli* HslV. In addition, *hsp20* gene was cloned from *H. pylori* strain NCTC 11637, expressed, and characterized.

Materials and Methods

Strains and Plasmid

Helicobacter pylori NCTC11637 was used in this study. *E. coli* Top10 and BL-21(DE3) pLysS were needed for amplification of plasmid DNA and recombinant protein expression, respectively. *H. pylori* was cultured in brain heart infusion (BHI, Oxoid, Basingstoke, Hampshire, UK) broth supplemented with 0.4% yeast extract and 10% horse serum (Gibco, Madison, WI) and incubated at 37°C in an atmosphere of 5% CO_2 in a humidified CO_2 incubator (Forma Scientific, Marietta, OH). Plasmid transformed *E. coli* was grown in LB medium supplemented with 50 μg/ml ampicillin at 37°C. The expression vector used was pET16b (Novagen, Madison, WI).

DNA Isolation, Amplification, and Sequencing

Genomic DNA of *H. pylori* was isolated according to the method described by Hua et al. [13]. Primers were designed according to HslV of *H. pylori* genomic sequence [12]. The forward primer used was 5′-AAAGGATCCGTTTGAAGCGACGACG-3′ while the reverse primer used was 5′-AAAGGATCCTTAAAGCTCCAAAATTTTAATATT-3′. Two *Bam*HI restriction sites were introduced in both 5′ and 3′ for insertion into the pET16b expression vector. The amplification conditions were set for denaturation at 94°C for 5 minutes followed by 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds and with additional extension at 72°C for 10 minutes. The PCR amplified gene fragment of 543 bp was inserted into expression vector pET16b at *Bam*HI restriction site. *Xho*I and *Ssp*I restriction enzyme digestion [14] provided information on gene orientation in the recombinant plasmid. The correct recombinant pET-hsp20 was sequenced using BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, Wellesley, MA) in ABI 100 model 377 DNA sequencer (Perkin Elmer).

Recombinant Protein Expression and Purification

The recombinant pET-hsp20 was transformed into BL-21 (DE3) pLysS and induced with IPTG (0.4 mg/ml) at each time interval. The expressed recombinant HSP20 protein (rHSP20) was found to exist as an inclusion body in host cells. Upon achieving the maximum expression, cells were harvested at 5000 × *g* for 5 minutes at 4°C and re-suspended in 20 mM phosphate saline buffer (PBS) and sonicated for 10 × 15 seconds at 5 MHz amplitude in a Soniprep 150 sonicator (Sanyo, Watford, UK). The inclusion body was collected by centrifugation at 10,000 × *g* for 10 minutes at 4°C, washed twice with PBS buffer and dissolved in 6 M Urea PBS. Purification of the inclusion body was carried out using affinity chromatography through a Nickel chelating column under denatured conditions. Recombinant protein was refolded through dialysis with 20 mM PBS. Protein concentration and purity were evaluated by Bio-rad Protein Assay. The purified protein band on SDS-PAGE was excised and analyzed by ESI Nanospray Ionization tandem MS on Micromass Q-TOF-2™ (Milford, MA).

Antibody Preparation

An antibody against rHSP20 was raised as described by Coligan et al. [15]. The study has been approved by the Animal Experimental Ethic Committee, National University of Singapore. Purified recombinant HSP20 protein was injected into New Zealand white rabbits with two different dosages, 120 μg and 150 μg. A rabbit injected with PBS buffer alone served as the control. Serum was separated from whole blood and antibody titer was assayed by indirect ELISA [16] method using 0.5 μg purified rHSP20 as antigen.

Antibody Purification and Characterization

Protein A sepharose CL-4B (Pharmacia, Uppsala, Sweden) affinity column was used to purify IgG from antiserum [15]. The serum was diluted with 50 mM Tris-Cl buffer (pH 7.0) and loaded into the column. The specific IgG was eluted with 0.1 M glycine-Cl buffer pH 3.0 and neutralized with 1 M Tris-Cl pH 9.0 (50–100 μl/ml fraction) right after elution. The specificity of antibody was verified using Western blotting on different protein extracts of *H. pylori* [16].

H. pylori *NCTC11637 Total Protein Extraction*

The following methods were used for the extraction of various protein fractions.

1 *Modified acid-glycine extraction* [17] In brief, 3-day-old-culture grown in BHI broth supplemented with 0.4% yeast extract and 10% horse serum was harvested and lysed using 0.2 M acid glycine (pH 2.2). Supernatant collected was dialysed against PBS at 4°C overnight. The acid-glycine extraction (AGE) comprises mainly cell membrane and membrane associated proteins. The protein concentration of the dialysate was measured by Bio-rad Protein Assay. Aliquots of 5 µg and 15 µg total proteins ran on SDS-PAGE were used for silver staining and Western blotting analyses. The linear pH 5–8 IEF strips (Bio-rad) were chosen based on the ProtParam tool, which shows HSP20 was a preponderance for pI ~6. All the 2D gel processing steps were carried out according to the manufacturer's instructions [18].

2 *Lysis method* The *H. pylori* cells were lysed according to the method described by Berkelman and Stenstedt [18]. The 3-day-old cultures were washed three times with ice-cold PBS and resuspended in lysis buffer containing 9 M Urea, 4% CHAPS, 40 mM Tris-Cl pH 8.8, protease inhibitor cocktail (Roche, Basel, Switzerland), and freshly prepared 50 mM DTT for 10^9 cells in each volume of 300 µl–400 µl. The cell suspension was left on ice for 1 hour and was vortexed occasionally. DNase (20 U) and RNase (20 U) were added into the cell suspension and left on ice for 10 more minutes before centrifuging at 10,000 × *g* for 10 minutes at 4°C. The resultant supernatant was transferred into a new eppendorf tube and stored at −80°C until use. The lysed fraction consists primarily of total protein of *H. pylori.* The protein concentration and the amount of protein for running SDS-PAGE were the same as for AGE protein.

3 *Outer membrane protein (OMP) extraction* OMP was isolated according to the method described by Ascencio et al. [19]. In brief, harvested 3-day-old cells were broken by sonication (30 seconds × 3). Unbroken cells were removed by centrifugation at 5000 × *g* for 30 minutes at 4°C. The supernatant was further centrifuged at 20,000 × *g* for 90 minutes at 4°C and the pellet was suspended in 150 µl of distilled water. Samples were treated with eight volumes of 2% sodium N-lauroylsarcosine for 1 hour at room temperature. The insoluble OMP was pelleted twice by centrifugation at 20,000 × *g* for 60 minutes at 4°C and washed twice with 2 ml deionized water to remove the excess detergent. Finally, the pellet was resuspended in 50 µl of distilled water, and subjected to SDS-PAGE and Western blotting analysis [14].

4 *Cytoplasmic protein (CP) extraction* A cytoplasmic fraction of *H. pylori* cells was extracted according to Chmiela et al. [20]. Briefly, the 3-day-old *H. pylori* culture was harvested and the cell pellet was resuspended in an appropriate volume of PBS buffer (~10^9cells/ml), followed by sonication (30 seconds × 3). The cell suspension was centrifuged at 20,000 × *g* for 90 minutes at 4°C. The resultant supernatant was collected and sterilized by filtration through a 0.2 µm filter (Sartorius, Goettingen, Germany). The protein concentration was measured by Bio-rad protein assay. 15 µg protein was subjected to SDS-PAGE and Western blotting using the rHSP20 antibody as a probe [14]. HSP60 antiserum (kindly provided by Lund University, Sweden) was used as an internal control.

Immuno-Gold labeled Transmission Electron Microscopy (TEM)

Pre-embedding labeling was employed for immuno-gold labeled TEM as described by Polak et al. [21]. The purified polyclonal rabbit IgG against rHSP20 (25 µg/ml) was used as the primary antibody, while the second antibody used was 10 nm or 20 nm gold-Protein A (1 : 20 dilution). *H. pylori* NCTC 11637 3-day-old cultures were used. The harvested cells were washed with PBS and fixed with 4% paraformaldehyde, 0.1% glutaraldehyde, with or without Triton X-100 (0.5%). Neutralization was done by incubating in 0.05 M glycine-PBS for 15 minutes and washing with PBS. The fixed cells were blocked with 0.5% BSA-PBS buffer and then conjugated with the primary antibody at 37°C for 2 hours. They were then reacted with the secondary antibody at 37°C for an additional 2 hours. The Immuno-gold labeled cells were further fixed in 2% glutaraldehyde at room temperature for 2 hours. The fixed immuno-gold labeled cells were processed through dehydration, infiltration and embedding in Low Viscosity Epoxy Resin (LVER) (Agar Scientific, Stansted, UK). Ultra-thin sections were viewed under the Philips 208S electronic microscope (Einhovery, Netherlands). Triton X-100 (0.5%) [22] was used to partially solubilize the cell

membrane for 10 or 20 minutes during the fixation step before applying 5 nm and 10 nm gold-Protein A as the secondary antibody. The preimmune serum and secondary antibody addition alone were used as negative controls. In testing the effectiveness of solubilization of the cell membrane using Triton X-100 treatment, *E. coli* cells labeled with anti-rHSP20 (25 µg/ml) were used as the negative control, while anti-HSP60 antiserum (1 : 20) labeled *H. pylori* was used as the internal control.

Antibody Against rHSP20

rHSP20 was used as an antigen to detect, by indirect ELISA, the presence of antibody against HSP20 in sera obtained from patients with different gastroduodenal diseases [23]. Briefly, 0.5 µg of rHSP20 was coated onto the 96-well ELISA plate and incubated at 4°C overnight. Aliquots of 100 µl of 1 : 50 diluted sera in PBST were added per well in triplicate for each serum sample. Horseradish peroxidase (HRP)-conjugated mouse antihuman IgG (DAKO, Glostrup, Denmark) was added as the secondary antibody. Substrate (0.4% O-phenylenediamine dihydrochloride) was used and the enzymatic reaction was stopped by the addition of 2 M H_2SO_4. The result was read at OD_{492} in a Labsystems Multiskan Ascent microtitre plate reader (Vantaa, Finland). In this study, the disease status of the patients was confirmed earlier by histology and serology. A total of 25 and 32 sera samples from patients with peptic ulcer and nonulcer dyspepsia, respectively, were examined. Sera from 32 normal subjects without any gastroduodenal complaint served as the negative control. All samples were randomly selected for this study. Data obtained were analyzed by statistical *t*-test.

Results

Construction of Recombinant Expression Vector

The PCR amplified target 543 bp (Figs 1A and 2A) was fused with 10 Histidine-tag in expression vector pET16b at *Bam*HI site giving a total length of 6255 bp (Fig. 1B). The clone with the correct orientation was shown to have a 533-bp fragment (Fig. 2B).

Recombinant Protein Expression and Purification

The estimated molecular weight of expressed protein is ~23kDa (Fig. 3). The maximum

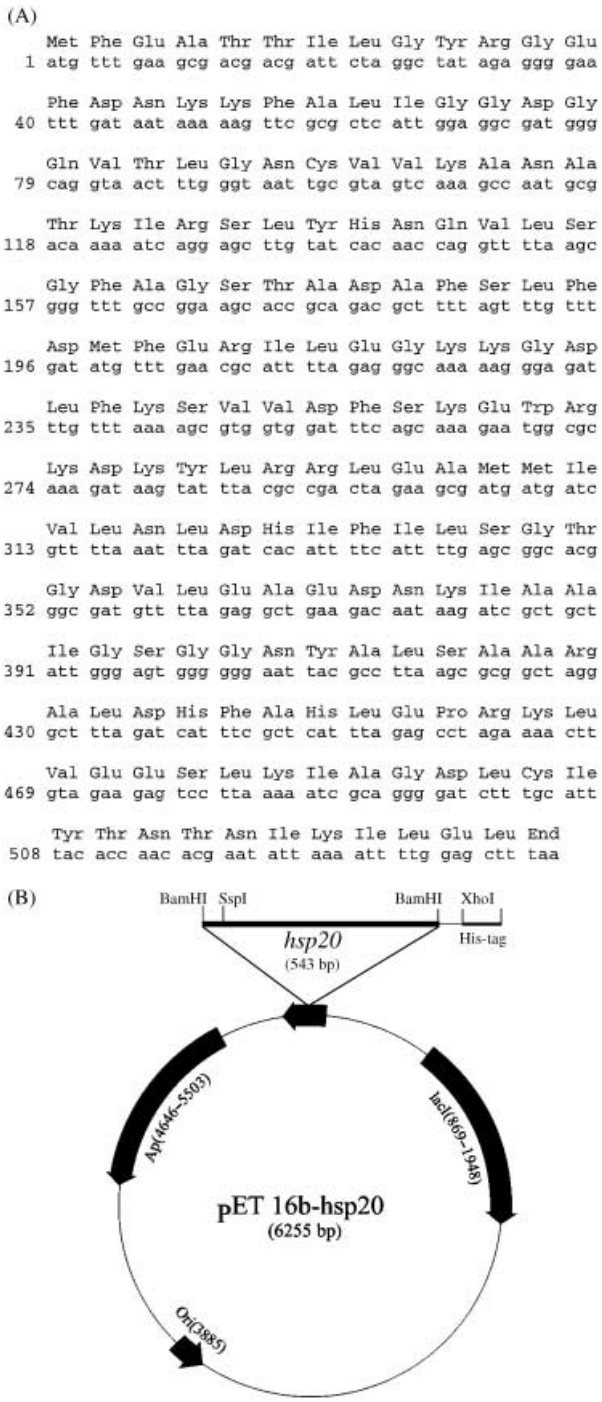

Figure 1 (A) The DNA and amino acid sequence of *Helicobacter pylori* NCTC11637 HSP20. The full length of *hsp20* is 543 bp and 181 amino acids. (B) Construction of the recombinant expression vector.

expression level was achieved at 3 hours upon induction with 0.4 mM IPTG (Fig. 3). There was no significant difference shown with 0.2 mM IPTG induction (data not shown). The expressed protein existed as an inclusion body

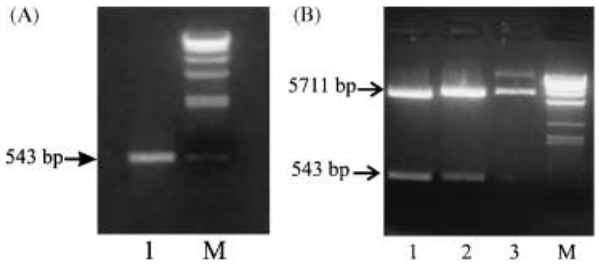

Figure 2 (A) PCR amplified hsp20 gene fragment. M, λ *Hin*dIII DNA marker; lane 1, target gene fragment (543 bp). (B) Recombinant pET16b-*hsp20* plasmid. Lane 1, recombinant plasmid (pET16b + *hsp*20, 6255 bp); lane 2, recombinant plasmid digested with *Bam*HI, a digested fragment of 543 bp; lane 3, recombinant plasmid digested with *Xho*I + *Ssp*I giving a fragment of 533 bp; M, λ *Hin*dIII DNA marker.

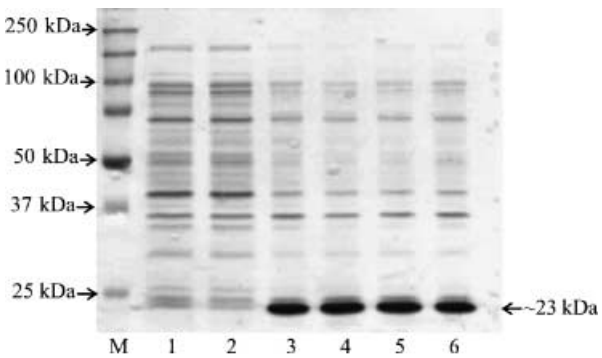

Figure 3 Expression rHSP20 in *E. coli* BL-21. Lanes 1 & 2, proteins of un-induced cells; lanes 3 & 4, IPTG induction for 3 hours; lanes 5 & 6, IPTG induction for 4 hours; M, Prestained Precision Protein Standards (Biorad, Hercules, CA).

(Fig. 4). The recombinant protein rHSP20 was eluted from His-tag affinity chromatography column with gradient imidazole at the concentration range of 0.3–1 M (Fig. 4). The percentage of expressed protein in total cell extracts ranged from 15%–20%, and recovery efficiency of inclusion body is about 20%. Through Q-TOF analysis, the amino acid sequence of recombinant rHSP20 was identified as heat shock protein HslV (HP0515) [12].

Antibody Preparation and Characterization

The antibody titre raised in rabbits against rHSP20 was over 1 : 12,800 regardless of the dosages used (120 µg and 150 µg). However, it was shown that immunizing with 120 µg produced higher antibody titre (data not shown). Western blotting shows an intense protein band of ~18 kDa in AGE preparation compared with proteins extracted by lysis buffer. The outer membrane protein fraction loaded was below the measurable level using Biorad protein assay, however, a specific band of similar molecular weight was observable in WB (Figure 5A,B). Similarly, in the WB of cytoplasmic fraction, a dim band of ~18 kDa showed up (Figure 6). In contrast, when HSP60 antiserum was used as the internal control probe, a band of slightly > 50 kDa was detected in both AGE and cytoplasmic fractions (Figure 6). Interestingly, two-dimensional gel electrophoresis (2D) also showed up a specific protein spot at pH of ~5.5 with a molecular weight of ~18 kDa (Figure 7).

Immuno-Gold Labeled TEM

Immuno-gold particles of 10 nm and 20 nm were seen to localize on the cell surface of *H. pylori* (Figure 8C,D). With Triton X-100 treatment, the cell membrane was partially broken; hence the gold particles were also observed on the remnants of cell membrane (Figure 9). In contrast, no gold particles were observed on the two negative controls of preimmune serum incubation and gold-Protein A (Figure 8A,B). Similarly, in

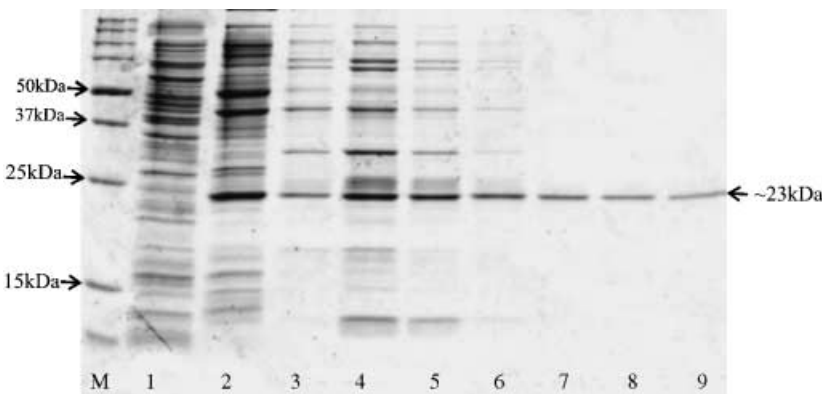

Figure 4 SDS-PAGE (15%) of purified rHSP20. Lane 1, supernatant of sonicated cells; lane 2, inclusion body solubilized by 6 M Urea; lanes 3–9, fractions eluted from His-tag column in ascending gradient with concentration of imidazole range from 0.3 to 1 M (inclusion body solubilized by 6 M Urea); M, Prestained Precision Protein Standards.

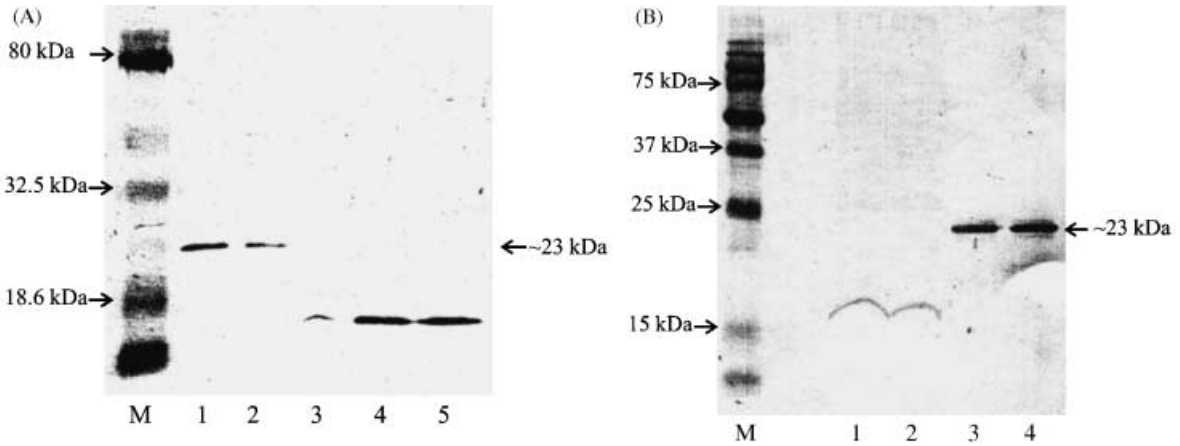

Figure 5 (A) Western Blotting of different cell fractions of *Helicobacter pylori*. Lanes 1 & 2, purified rHSP20 protein (used as positive control); lane 3, *H. pylori* outer membrane protein fraction; lanes 4 & 5, *H. pylori* acid glycine extract; M, Kaleidoscope Polypeptide Standards (Biorad). (B) Western Blotting of *H. pylori* total cell proteins extracted by lysis buffer. Lanes 1 & 2, *H. pylori* cells total proteins extracts; lanes 3 & 4, purified rHSP20 protein (positive control); M, Prestained Precision Protein Standards (Biorad).

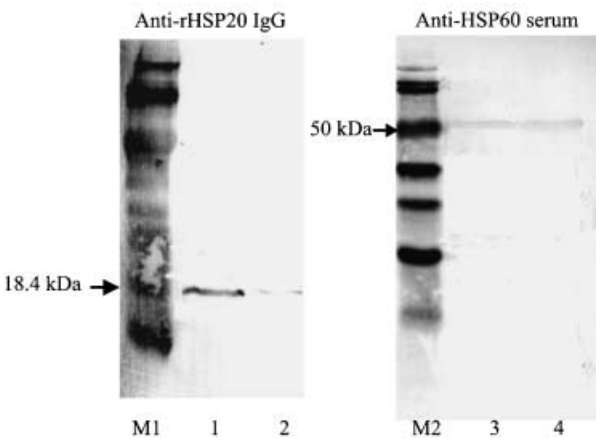

Figure 6 Western Blotting of different subcellular fractions of *Helicobacter pylori* using different probes. Lanes 1 & 3, acid glycine extract (AGE) of *H. pylori*; lanes 2 & 4, cytoplasmic fraction (CF) of *H. pylori*; M 1, Kaleidoscope Polypeptide Standards; M 2, Prestained Precision Protein Standards. Lanes 1 & 2 were probed with rHSP20 antibody; lanes 3 & 4 were probed with *H. pylori* – HSP60 antiserum.

Triton X-100 treated *E. coli* cells, there was no gold particle observed (Figure 10A). However, when Triton X-100 treated *H. pylori* cells were labeled with HSP60 antiserum, gold particles were observed mainly in the cytoplasmic regions with some on the surface (Figure 10B).

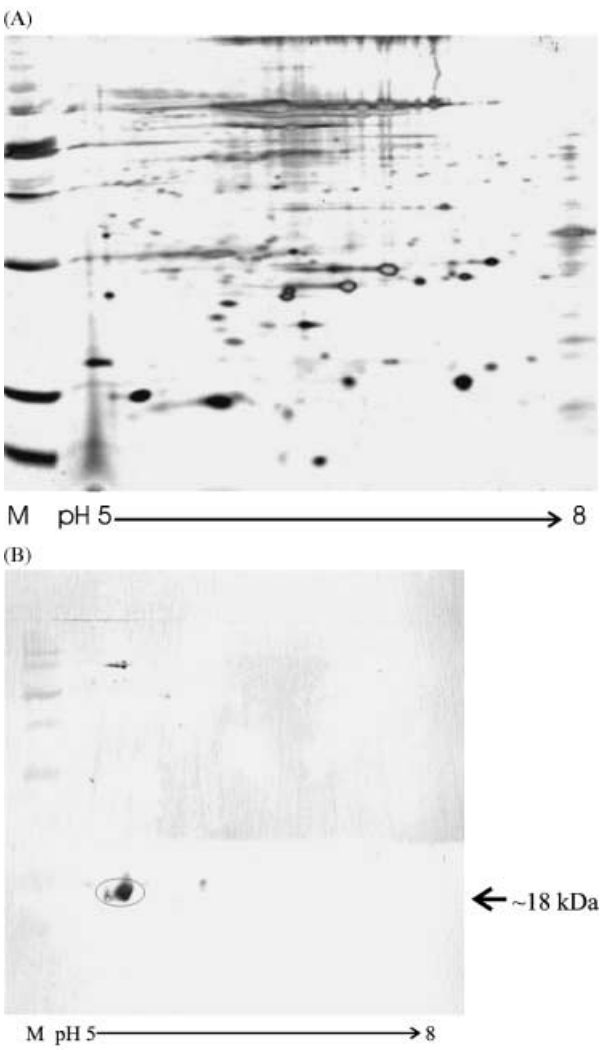

Figure 7 Two-dimensional gel electrophoresis and Western blotting of acid glycine extract of *Helicobacter pylori*. (A) 2-D gel of AGE silver staining; (B) Western blot of 2-D gel of AGE; M, unstained Precision Protein Standards.

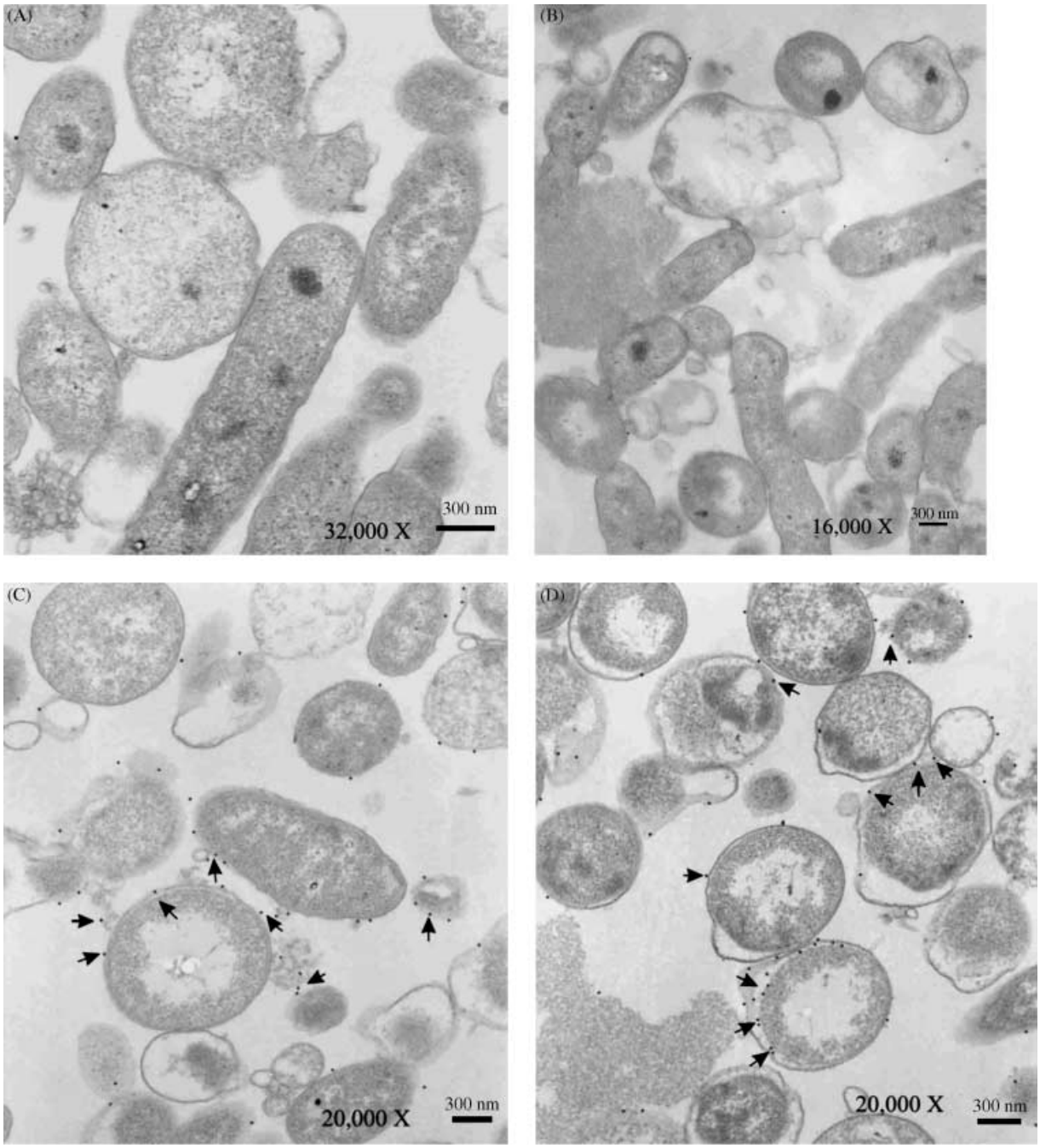

Figure 8 Transmission Electron Microscopy of *Helicobacter pylori* NCTC 11637 cells labeled with different primary antibodies and different size of immuno-gold particles. (A) negative control – with immuno-gold only; (B) negative control – with preimmune rabbit IgG; (C) antirHSP20 IgG + 10 nm immuno-gold; (D) anti-rHSP20 IgG + 20 nm immuno-gold; Arrows show sites of localization of HSP20.

Antibody titre against HSP20

It is interesting to note that there was no significant difference ($p > 0.3$) between the antibody level against rHSP20 in the control subjects and patients with gastroduodenal diseases. In the disease group, the antibody level against rHSP20 was in the same titre for all patients as well as the normal subjects tested (Table 1).

Discussion

The amino acid sequence of HSP20 of *H. pylori* 11637 shows 96% and 97% identity to HslV of

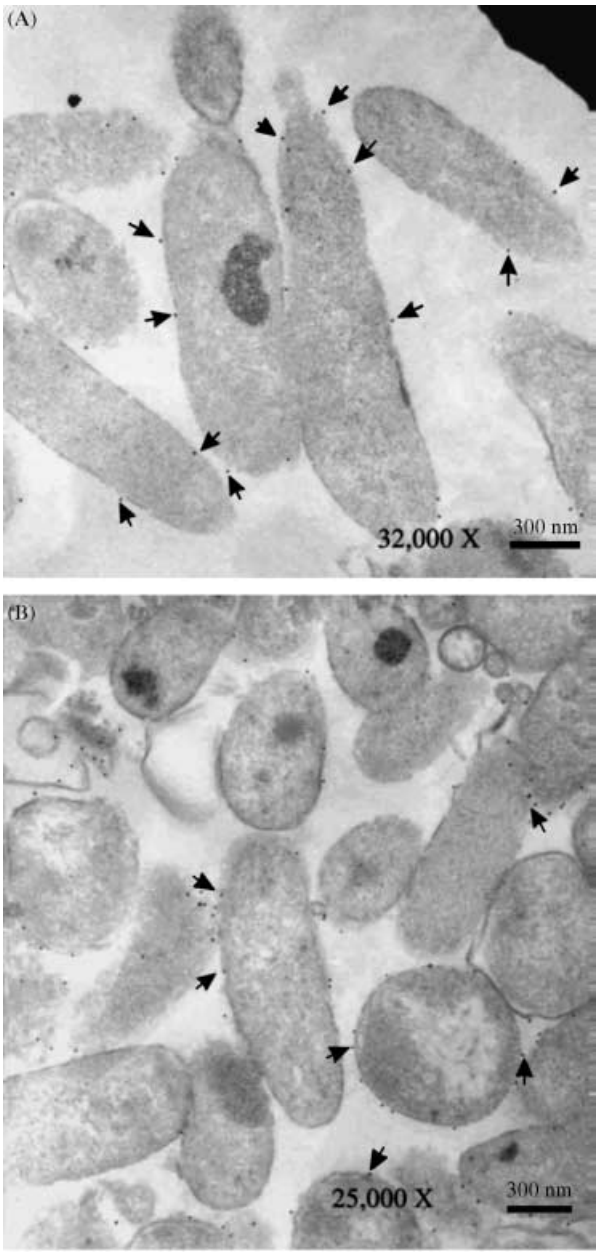

Figure 9 Transmission Electron Microscopy of *Helicobacter pylori* NCTC 11637 cells labeled with anti-rHP20 IgG and 10 nm Immuno-Gold after Triton X-100 treatment. (A) anti-rHSP20 IgG + 10 nm immuno-gold + 10 min Triton X-100 treatment; (B) anti-rHSP20 IgG + 10 nm immuno-gold + 20 min Triton X-100 treatment; Arrows show site of localization of HSP20.

H. pylori strains 26695 [12] and J99 [24], respectively. It also shows 48–49% identity with HslV of *E. coli* and *Bacillus subtilis*. Its homologue, HslV of *E. coli* was determined to be a component of ATP-dependent protease serving as a peptidase [25]. Based on the protein sequence analysis and peptidase activity (data not shown), it is probable that *H. pylori* HSP20 has a function of peptidase activity similar to that of HslV of *E. coli* [7].

In this study, where rHSP20 antibody was used as a probe in Western blotting of various fractions of *H. pylori*, a specific and intensive band of ~18KDa was lighted up in acid glycine extract that comprises mainly membrane and membrane-associated proteins, as well as in the outer membrane fraction (Figure 5A,B). However, one dim band of ~18 kDa was detected in the cytoplasmic fraction. This implies that HSP20 is mainly localized on the cell surface of *H. pylori*. The surface localization was further confirmed by immuno-gold labeled TEM, where both 10 nm and 20 nm gold particles were found to locate mainly on the bacterial cell surface (Figure 8C,D). Furthermore, it was shown that after Triton X-100 treatment, where cell membrane was partially solubilized, the gold particles were shown to attach mainly to the cell membrane or remnants of the cell membrane of *H. pylori* (Figure 9). However, in Triton X-100 treated *E. coli* cells no gold particle was observed, which indicates that the rHSP20 antibody is more specific to *H. pylori.* The results of the internal control, where Triton X-100 treated *H. pylori* labeled with HSP60 antiserum, show that gold particles were mainly localized in the cytoplasmic regions and agree with a previous study [26]. Both controls demonstrate that Triton X-100 treatment is effective in membrane solubilization for assisting in the penetration of antibody during the immunolabeling. The results support the surface localization of HSP20 in *H. pylori*.

It is highly significant that only a single protein spot of ~18 kDa at pH ~5.5 was detected on the WB of 2-D gel of acid glycine extract when rHSP20 antibody was used as a probe. It further supports 2-D gel electrophoresis being an efficient method for analysis of complex protein mixtures extracted from cells [18].

The antibody level to HSP20 in patients with gastric diseases and the control subject was found to cluster within a close range (0.21 ± 0.02). This could possibly be due to a cross-reaction resulting from earlier exposure to *H. pylori* by both control subjects and younger patients. This might have resulted in a low but constant IgG level of HSP20. This is possible as the association of *H. pylori* with humans was reported to have begun at the early age of 10 [26], while the tested subjects in this study were all aged above 20.

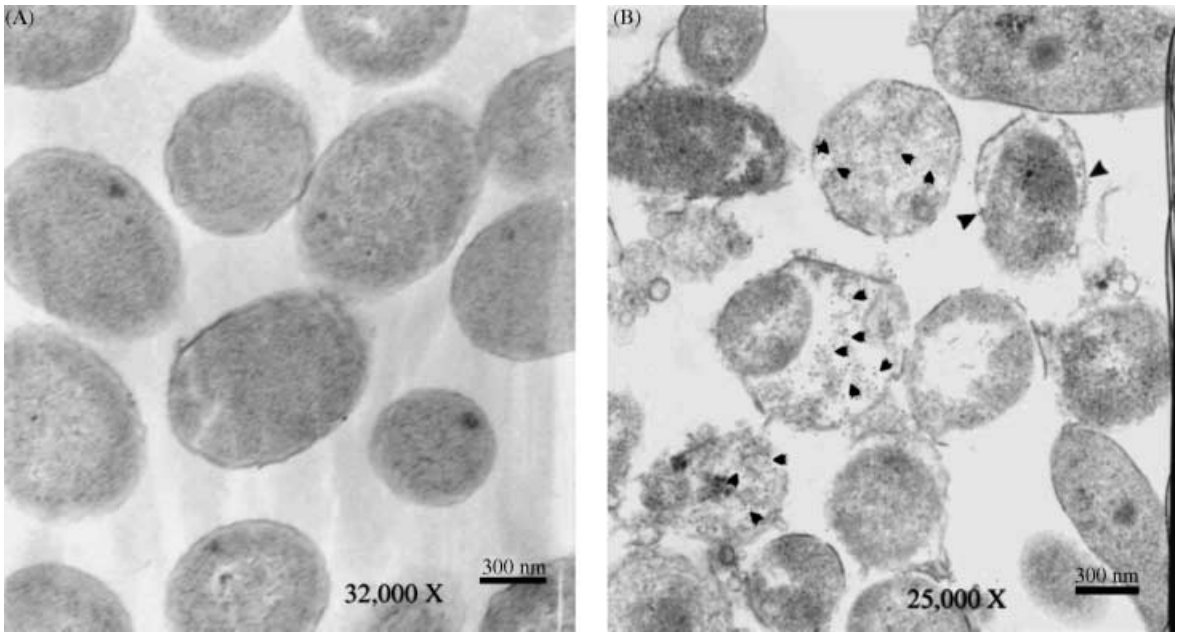

Figure 10 Two controls of immunolabeled Transmission Electron Microscopy. Cells treated with Triton X-100 for 20 minutes and 5 nm immuno-gold. (A) *E. coli* cells labeled with rHSP20 antibody; (B) *Helicobacter pylori* labeled with *H. pylori* – HSP60 antiserum (big arrow showing the surface localization, small arrow showing the cytoplasmic localization).

Table 1 Sero-prevalence of patients with different gastro-duodenal diseases with or without *Helicobacter pylori* infection*

	Gastro-duodenal diseases				
	PUD		NUD		Normal
H. pylori histology[1]	Positive	Negative	Positive	Negative	ND
OD_{492} & (CI)[2]	0.211 (0.191–0.232)	0.216 (0.202–0.230)	0.220 (0.190–0.249)	0.231 (0.228–0.233)	0.234 (0.195–0.259)
No. of cases	17	8	17	15	32
p for trend[3]	0.661	0.661	0.324	0.324	0.574

*Purified rHSP20 was used as antigen for detection by ELISA.
PUD, duodenal ulcer and gastric ulcer, NUD, gastritis and nun-ulcer dyspepsia; ND, not done.
[1]Positive or negative status for histology. [2]Optical density at 492 nm and 95% confidence interval of OD value. [3]All *p* values are from two sided tests.

It is interesting that the ELISA readings cluster within a low range of 0.21 ± 0.02. This is unlike the wide OD range between patients with gastro-duodenal diseases and control subjects when other antigens were used [28]. This may indicate that HSP20 does not play the vital role in the pathogenesis of *H. pylori* infection, unlike HSP60, which has been reported by Yamaguchi et al. to be involved in chronic gastric inflammation following *H. pylori* infection in man [3].

It is common for bacteria pathogens to colonize prior to pathogenesis [29]. As HSP20 is localized on the cell surface of *H. pylori,* it may be involved in assisting colonization of the organism on the host. Following colonization, other bacterial proteins like CagA and VacA may function as pathogenic factors leading to *H. pylori* infections [30,31]. Hence, the low antibody titer to HSP20 was shown in both control subject and patients. Many surface proteins of bacteria play an important role in pathogenesis through adhesion followed by bacterial colonization leading to initiation of immune response [2]. As HSP20 is expressed on the cell surface of *H. pylori* and the low antibody titer detected in human hosts, its role in adhesion similar to HSP60 cannot be excluded. The importance of HSP20 cannot be undermined as adhesion is an important process in the course of pathogenesis. The homology of the primary structure of HSP20 and human β-type subunits of 20S proteosome (a cytoplasmic protein) is 34%. Preliminary study using the method described by Seong et al. [7] showed that HSP20 has weak peptidase activity (unpublished data). Further

study on this surface protein may help to elucidate the importance of surface proteins like HSP20 in bacterial–host interaction.

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Heat Shock Protein 20 as a potential colonization factor and chaperon of CagA in *Helicobacter pylori* infection in mice

Short title: HSP20, a colonization factor and chaperon of CagA

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Abbreviations: AGE: acid glycine extract; BHI: brain heart infusion; CBA; chocolate blood agar; CO-IP: co-immunoprecipitation; ELISA: enzyme-linked immunosorbent assay; FITC: fluorescein isothiocynate; HRP: horseradish peroxidase; HSP20: heat shock protein 20; MALDI-TOF: matrix-assisted laser desorption/ionization-time of flight mass spectrometry; OMP: outer membrane protein; ORF: open reading frame; rCagA: recombinant CagA protein; rHSP20: recombinant heat shock protein 20; RT-PCR: reverse-transcriptase polymerase chain reaction; SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TP: total protein; WB: western blotting.

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Abstract

Background & Aims: Surface molecules of *H. pylori* are crucial for the adhesion of the organism on the host gastric epithelial cells. The potential relevance of a newly identified surface localized heat shock protein 20 (HSP20) to colonization and the pathogenesis of *H. pylori* infection was explored.

Methods: From a mouse adapted strain *H. pylori* SS1, a *hsp20*-isogenic mutant was constructed using homologous recombination. The adhesion and colonization ability of the isogenic mutant was analyzed by *in vitro* and *in vivo* studies. Using reverse-transcriptase polymerase chain reaction (RT-PCR) and co-immunoprecipitation, the relationship between HSP20 and the pathogenesis of *H. pylori* infection was postulated.

Results: *In vitro* adhesion assay showed that there was no significant difference between the adherence ability of wild type and *hsp20*-isogenic mutant onto the two gastric cell lines (Kato III and AGS). However, unlike the wild type *H. pylori* SS1, the isogenic mutant lost its ability to colonize the stomachs of BALB/c mice. RT-PCR analysis revealed that HSP20 functioned independently from the major adhesins like OipA, HopZ and SabA. Interestingly, co-immunoprecipitation test demonstrated a novel and unique interaction between HSP20 & CagA. Further analyses uncovered a strong association between HSP20 and the surface presentation of CagA in *H. pylori*.

Conclusions: The inability of *hsp20*-isogenic *H. pylori* to colonize in the mice suggests the potential involvement of HSP20 in colonization. In addition, the surface presentation of CagA resulting from the unique interaction between HSP20 and CagA highlights the novel chaperonic role of HSP20 for CagA in *H. pylori* infection.

Introduction

H. pylori infection has been found to be strongly associated with various gastroduodenal diseases that affects half of the world population (1). Many virulence factors (e.g. CagA, VacA) have been identified in the past two decade (2), however, the exact pathogenesis of *H. pylori* has not been well established. As an extracellular pathogen, the interaction between *H. pylori* and host plays an important role in *H. pylori* infection.

In *H. pylori* infection, bacterial adhesion and colonization on gastric epithelial cells will be the prerequisite in successful establishment of the bacteria on mucus layer of the host (3). Many surface molecules of *H. pylori* have been reported to be involved in this process. Among which, urease was reported to be able to decompose urea into ammonia to buffer the acidic pH in the stomach (4); flagella could efficiently propel the bacteria through viscous mucus layer (5) and different adhesins (e.g., BabA, OipA, HopZ and SabA) could bind to the specific receptors on the epithelial cells (6; 7). In addition, cytoplasmic proteins (e.g., HSP60, SOD) were specifically translocated onto the bacterial surface to combat against the hostile conditions in host (8; 9). Although these different molecules may function discretely, they are integrated and dependent on or cooperate with each other in the pathogenic process of *H. pylori* infection. Thus, many recent studies have focused and addressed on the relationship between bacterial surface proteins and pathogenesis of *H. pylori* infection (10; 11).

To date, numerous isogenic *H. pylori* mutants constructed have shown the inability to colonize in the stomachs of animals, suggesting the correlation of these protein molecules to the colonization of *H. pylori*. These proteins included urease (12; 13), γ-glutamyl transpeptidase (GGT) (14; 15) and flagella (16; 17). However, controversial results have

been reported from different research groups in regards to the colonization of *H. pylori in vivo*. This may indicate that the process of *H. pylori* adhesion and colonization is not merely the outcome of a single molecule but the consequence of interactions of multiple factors. Thus, studying on individual gene may help to resolve the different function of each gene and the understanding on the cooperate interactions between genes in the initial process of *H. pylori* infection.

As a new member of heat shock protein family, heat shock protein 20 (HSP20) has been identified to be located on the cell surface of *H. pylori* (18). However, antibody against HSP20 in the sera of patients was low and insignificant as compared to that of the normal subjects (18). These findings highlight that HSP20 might not be involved in the induction of host immune responses during *H. pylori* infection but could assist in the adhesion and most probably in the colonization of the organism on the host gastric epithelial cells. Hence, in order to investigate the relevance of HSP20 to bacterial adhesion or colonization, the study proposes a collaborative role of HSP20 in the pathogenesis of *H. pylori* infection following adhesion/colonization.

Materials and Methods

Bacterial strains and cell lines

Helicobacter pylori SS1 (mouse adapted strain) was used in this study. *H. pylori* cells were grown either on chocolate blood agar plate (CBA) supplemented with antibiotics (19) or in the brain heart infusion (BHI) broth (Oxiod, Hampshire, UK) supplemented with 0.4% yeast extract (Oxiod, Hampshire, UK) and 10% horse serum (Gibco, Calsbad, CA, USA). LB broth and agar plate with or without supplementation of 50 μg/ml Ampicilin (Sigma, St. Louis, MO, USA) and/or 25 μg/ml Kanamycin (Sigma, St. Louis, MO, USA) was used for the growth of *E. coli* Top10. Gastric carcinoma cell lines: Kato III and AGS (ATCC, Manassas, VA, USA) were grown in RPMI medium (Sigma, St. Louis, MO, USA) supplemented with 10% fatal bovine serum (Gibco, Calsbad, CA, USA).

Construction of *hsp20::aphA* gene-targeting vector

Plasmid pBluescript II SK (+) and the kanamycin resistant gene (*aphA*) from plasmid pILL600 (kindly provided by A. Labigne Pasteur Institute, Paris, France) were chosen for the construction of *hsp20::aphA* gene-targeting vector. Four primers (KO1, 2, 3 & 4, Table 1) were designed according to the known *H. pylori* 26695 DNA sequences (20) for the amplification of two flanking DNA sequences (5' & 3') of *hsp20*. Genomic DNA of *H. pylori* SS1 was used as the template for the amplification. The schematic construction of targeting vector is as shown in Fig 1. PCR amplification was performed by an initial denaturation at 94°C for 5 minutes followed by 94°C, 30 seconds; 50°C, 1 minute; 72°C, 1 minute 30 seconds for 30 cycles with additional extension at 72°C for 10 minutes. The 5' flanking fragment (2700 bp) was inserted at BamHI & PstI sites while 3' fragment

(2549 bp) was ligated at SalI & ApaI sites of the vector. The 1340 bp long *aphA* gene fragment was inserted at EcoRI site to disrupt *hsp20* gene in the vector. The positive recombinant plasmid (pBS-5'-*aphA*-3') was screened using the corresponding restriction enzyme digestion and confirmed by DNA sequencing.

Homologous recombination of *hsp20::aphA* in *H. pylori*

Natural transformation was used for the homologous recombination between *hsp20::aphA* gene-targeting vector and *H. pylori* genome. The protocol for natural transformation of *H. pylori* with plasmid DNA (pBS-5'-*aphA*-3') was carried out as described by Heuermann and Hass (21). The colonies grown on the kanamycin containing CBA plate were selected and sub-cultured for a few rounds to purify *hsp20::aphA* transformed *H. pylori* mutants. The purified *hsp20*-isogenic *H. pylori* mutant was preserved in BHI broth medium supplemented with 10% horse serum and 20% glycerol and stored at - 80°C for further analysis.

Identification of *hsp20*-isogenic *H. pylori* mutant

The identification of *hsp20*-isogenic *H. pylori* was performed as described by Goodwin *et al*. (22). The genomic DNA of *hsp20*-isogenic *H. pylori* was used for the analysis. PCR amplification using different primers (HSP20-F & R; Km-F & R; HSP20-F & Km-R; KO3 & T7, Table 1) and Southern blotting using different probes (*aphA* gene fragment & pBluscript plasmid DNA) were used to identify the insertion of *aphA* gene in the genome of the isogenic mutant. The standard protocols for Southern hybridization were followed as described in ECL Direct™ Nucleic Acid Labelling and Detection System by manufacturer (Amersham Biosciences, Uppsala, Sweden). The protein expression of HSP20 in the isogenic *H. pylori* mutant was further detected by Western blotting using

antibody against rHSP20 (0.5 μg/ml) (18) and goat anti-rabbit IgG HRP conjugate (1:2000 diluted) (DAKO, Glostrup, Denmark). Rabbit antiserum against *H. pylori* HSP60 (1:500 diluted) (18) was used as the internal control.

In vitro adhesion assay of _H. pylori_ to cell lines

The adhesion assay of *H. pylori* to human gastric carcinoma cell lines, Kato III and AGS, was performed according to the methods as described by Yamaguchi *et al*. (8). Either wild type *H. pylori* or *hsp20*-isogenic *H. pylori* mutant was added at different ratios of bacteria to cells (bacteria : cells = 50:1, 100:1 and 200:1). The adherent bacteria were analyzed using ELISA or flow cytometry methods (23; 24). The primary antibody used for detection was rabbit anti-*H. pylori* IgG (1:20000 diluted) (DAKO, Glostrup, Denmark) and the secondary antibodies used was goat anti-rabbit IgG HRP conjugate for ELISA or goat anti-rabbit FITC conjugate for flow cytometry (1:500 diluted) (Sigma, St. Louis, MO, USA).

Animal study of _H. pylori_ in mice

The animal study was approved by the Animal Experimental Ethic Committee, National University of Singapore and carried out according to the guidelines provided by Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC), U.S.A. Both wild type and *hsp20*-isogenic *H. pylori* SS1 strains were inoculated into BALB/c mice (15 – 20 g, < 4 weeks of age & single sex type). The inoculation procedure was performed according to the method as described by Smythies *et al*. (25). A total of 15 mice were inoculated for each *H. pylori* strain and 9 mice were fed with BHI broth alone serving as negative controls. At 2, 4 and 8 weeks after inoculation, mice were sacrificed, stomachs were removed and dissected longitudinally

into three equal parts for the detection of *H. pylori* by microbiological, immunohistochemistry and RT-PCR assays. Whole blood from the sacrificed mice was drawn and antibody responses to *H. pylori* were analyzed.

Acid glycine extract (AGE) of *H. pylori* SS1 and rHSP20 were used as antigens (0.5 μg/ml) to detect the antibody against *H. pylori* and the antibody against HSP20 respectively, in mice by ELISA method. The secondary antibody used was goat anti-mouse immunoglobulins HRP conjugate (1:2000 diluted) (DAKO, Glostrup, Denmark).

Gene status and expression of *H. pylori* adhesins

The gene status (in-frame or out-of-frame based on the CT dinucleotide repeats in the signal sequence) of three major adhesins, *oipA* (HP0638), *hopZ* (HP0009), *sabA* (HP0725) in both wild type and *hsp20*-isogenic *H. pylori* were examined using PCR amplification followed by DNA sequencing (26; 27). The presence of *babA2* gene was also analyzed using PCR (28).

Total RNAs of both wild type and the isogenic *H. pylori* were used to detect the expression of adhesin genes by RT-PCR. The amplification of 16s rRNA gene fragment of *H. pylori* was used as the internal control (29). The PCR amplifications for *oipA*, *hopZ* and *sabA* gene fragments were conducted by denaturation at 94°C for 5 minutes followed by 94°C, 30 seconds; 50°C or 52°C, 30 seconds and 72°C, 30 seconds for 35 cycles with additional extension at 72°C for 5 minutes. The primers used are included in Table 1.

Co-immunoprecipitation (CO-IP) and Western blotting

The procedure for CO-IP was performed according to Voland *et al*. (30). Antibody against rHSP20 (1 μg) (18) and an aliquot of 50 μl Protein A sepharose slurry were added for each reaction. The protein pulled down was separated on SDS-PAGE. The isolated

protein on the gel was identified by MALDI-TOF Mass Spectrometry at the Proteins and Proteomics Centre, National University of Singapore, Singapore. Four independent experiments were carried out.

Western blotting analysis using different antibodies was employed for further confirmation after CO-IP reaction. Mouse antiserum against rCagA (rCagA: his-tag fused CagA protein fragment corresponding 1st – 285th amino acids of *H. pylori* 26695, prepared by our lab, 1: 800 diluted) and antibody against rHSP20 (0.5 μg/ml) (18) were used as primary antibody respectively. CagA-negative *H. pylori* was served as negative control.

The expression of *cagA* in *H. pylori*

Total RNAs of *H. pylori* of different ages (3rd & 4th day) were used to detect the expression of *cagA* in both wild type and *hsp20*-isogenic mutant using RT-PCR (25). The transcribed DNAs were used as template for the amplification of *cagA* gene fragment (1st – 852nd, 852 bp) (20). The amplification of 16s rRNA gene fragment of *H. pylori* was used as the internal control (29). The primers used are included in Table 1.

The presence of CagA in *H. pylori* different sub-cellular factions

Different *H. pylori* cellular fractions (total protein, acid glycine extract and membrane fraction) were extracted from wild type, *hsp20*-isogenic mutant and CagA-negative *H. pylori* according to Du and Ho (18) before subjecting to SDS-PAGE analysis and Western blotting. The primary antibody used was mouse antiserum against rCagA while rabbit antiserum against HSP60 was used as the internal control. Similar tests were carried out in each of the cellular fractions obtained from the 3 different *H. pylori* cultures that were supplemented with the addition of 0.1, 0.5 or 1 μg/ml rHSP20.

Antibody against CagA in *H. pylori* inoculated mice

Antibody against CagA in both wild type and the isogenic *H. pylori* infected mice was analyzed using ELISA. rCagA [rCagA: his-tag fused CagA protein fragment corresponding 1^{st} – 285^{th} amino acids according to *H. pylori* 26695 (Ng Cheryl & Ho, unpublished)] at 0.5 μg/well was used as antigen for detection. The antigen used for the detection of antibody against Le (X) and (Y) were synthetic Le (X) and (Y) (IsoSep, Sweden) at 0.1 μg/well which served as internal controls. The procedure for the detection was carried out according to the protocols as described by Zheng *et al*. (31).

Results

Identification of *hsp20*-isogenic *H. pylori* mutant

Using natural transformation approach, a total of five *hsp20*-isogenic *H. pylori* SS1 mutants were obtained by homologous recombination. The insertion of kanamycin resistant gene (*aphA*) in *H. pylori* genomes of the isogenic mutants was identified by PCR amplification and Southern blotting hybridization (data not shown). The absence of HSP20 protein expression in the isogenic mutant was detected using Western blotting (data not shown).

***In vitro* adhesion assay of *H. pylori* to gastric cell lines**

The adhesion assay of *H. pylori* to Kato III and AGS cells analyzed using ELISA and flow cytometry showed that the adherence of *hsp20*-isogenic *H. pylori* to cells was 8 – 25% lower than that of wild type *H. pylori* (Table 2). However, there is no significant difference ($p>0.2$) in the adherence between *H. pylori* wild type and *hsp20*-isogenic mutant to the two cell lines *in vitro.*

Animal study of *H. pylori*

Analysis of mice gastric biopsy samples using microbiological, histological and RT-PCR showed that the wild type *H. pylori* started colonization in mouse stomach at 2 weeks of post-inoculation and persisted up to 8 weeks tested (Table 3). However, *hsp20*-isogenic *H. pylori* mutant was absent in stomachs of all mice tested throughout the course of inoculation (Table 3). The antibody against *H. pylori* in the wild type inoculated mice was significantly higher ($p<0.01$) than that in the isogenic mutant inoculated mice as shown in Fig 2A. However, there was no difference in the level of antibody against HSP20 in both *H. pylori* inoculated mice (Fig 2B).

Gene function status of *H. pylori* adhesins

The CT repeats (6 CT repeats in *oipA*, 7 CT repeats in *hopZ* and *sabA*) were consistent in both wild type and *hsp20*-isogenic *H. pylori* for each gene (Table 4). Based on the deduced amino acids in this region, it showed that ORFs of these genes were in-frame (i.e. "on" status) in both wild type *H. pylori* and the isogenic mutant. There was no *babA2* gene amplified in both wild type and the mutant *H. pylori*. Furthermore, RT-PCR showed that all the three genes (*oipA*, *hopZ* and *sabA*) were expressed in both wild type and *hsp20*-isogenic *H. pylori* (Fig 3A). As the internal control, the expression of 16s rRNA gene was detected in both wild type and the isogenic *H. pylori* (Fig 3B).

CO-IP and Western blotting

A specific protein band with molecular weight of 100-150 kDa was pulled down by antibody against rHSP20 from the total protein extract of the wild type *H. pylori* (Fig 4A) but was absent in *hsp20*-isogenic mutant. This specific protein was identified as the cytotoxicity associated immunodominant antigen (120 kDa, CagA, NCBI accession No.

P55746) (32) of *H. pylori* as identified by mass spectrometry MALDI-TOF. The matched peptides are shown in Fig 4B. The same results for CO-IP and MALDI-TOF were obtained in four independent experiments.

According to the results obtained from protein identification, Western blotting was carried out for CO-IP test. The protein isolated was recognized by mouse antiserum against rCagA (Fig 4C) as well as antibody against rHSP20 (Fig 4D).

CagA expression and partition in *H. pylori*

RT-PCR analysis showed that the gene fragment of *cagA* (1^{st} – 852^{nd}, 852 bp) was amplified in both wild type *H. pylori* and the isogenic mutant irrespective of ages of culture (Fig 5A) whereas the internal controls, 16s rRNA (390 bp) was detected in all *H. pylori* cultures (Fig 5B).

In the Western blotting analysis of different *H. pylori* sub-cellular fractions, it was found that CagA protein was detected in all fractions extracted (TP, AGE & OMP) of *H. pylori* wild type while CagA was only detected in TP and OMP fractions but absent in AGE fraction of *hsp20*-isogenic *H. pylori* mutant (Fig 6A). It was also noted that the intensity of CagA protein band detected in OMP fraction of the isogenic mutant is significantly lower than that of the wild type when equal amount of OMP protein was loaded. Interestingly, with the addition of rHSP20 in *H. pylori* cultures, Western blot showed that CagA protein was detected in all fractions (TP, AGE & OMP) of both wild type and the isogenic mutant. In addition, the intensity of CagA protein band detected in each fraction was similar in both *H. pylori* wild type and the isogenic mutant (Fig 6B) regardless of the various concentration of rHSP20 added. There was no CagA detected in all these different fractions of CagA-negative *H. pylori*. As the internal control, HSP60 was shown

to be present in all fractions of *H. pylori* wild type, the isogenic mutant and CagA-negative (Fig 6A & B).

Antibody against CagA in *H. pylori* inoculated mice

The antibody against CagA in the isogenic mutant infected mice detected was significantly lower ($p<0.05$) than that of the mice infected with wild type *H. pylori* (Fig 7A). However, the antibody against Le (X) and Le (Y) were insignificant between the mice infected with *H. pylori* wild type and the isogenic mutant (Fig 7B & C).

Discussion

The successful construction of *hsp20*-iosgenic *H. pylori* mutant has provided the opportunity to explore the potential role of HSP20 on *H. pylori* adhesion and colonization. The *in vitro* adhesion assays on AGS and Kato III cells showed no significant difference on the adherence ability of *hsp20*-isogenic *H. pylori* as compared to the wild type, even though there was a reduction (8% - 26%) in the adhesion capability of the isogenic mutant (Table 2). It indicates that *hsp20*-isogenic *H. pylori* retains almost similar adherence capability as that of the wild type. The slight decrease in adhesion capability of *hsp20*-isogenic *H. pylori* to AGS and Kato III cells under *in vitro* conditions implies that HSP20 is but one of the factors involved in the adhesion of *H. pylori* or that it may participate indirectly in the process of bacterial adherence. It therefore suggests and supports the role of various adhesins [e.g. BabA, OipA, HopZ, SabA and Le (X)] in *H. pylori* as reported by various studies on adhesion of *H. pylori* (7; 33-35). These multiple species of adhesins might function at different stages of bacterial infection or cooperate in tandem. This is an area that needs further investigation.

Colonization is the prerequisite for the pathogenesis of *H. pylori* (3). Interestingly, introduction of wild type *H. pylori* into mice showed successful colonization of organisms in the stomachs within 2 weeks post-inoculation and persisted up to 8 weeks. However, there was no colonization detected in the mice inoculated with *hsp20*-isogenic *H. pylori* throughout the course of the 8-week study (Table 3). The failure of *hsp20*-isogenic *H. pylori* to colonize in BALB/c mice indicates that HSP20 protein may be essential for bacterial colonization as exemplified by the significantly lower antibody level against *H. pylori* (but not against HSP20) in *hsp20*-isogenic mutant infected mice (Fig 2A). This low level of antibody against *H. pylori* could possibly be due to the unsuccessful establishment of the bacterial pathogen on gastric epithelium of the animals thereby further signifies the importance of HSP20 in bacterial colonization.

In contrast to the antibody level against *H. pylori*, there was no significant difference observed in the level of antibody against HSP20 between wild type, the isogenic mutant infected mice and the uninfected negative controls (Fig 2B). This may indicate that disrupted HSP20 in the isogenic *H. pylori* is not directly correlated with the low antibody production in the mutant infected mice. It could further attest that HSP20 is unlikely to be a major antigen that induces strong and consistent immune response during *H. pylori* infection. This result agrees with our previous finding where the antibody against HSP20 in patients with different gastroduodenal diseases was insignificant from that of the normal subjects (18).

The inability for *hsp20*-isogenic mutant to colonize in mice supports the many earlier findings that were carried out. This can be illustrated by the study of Dorrell *et al*. (1999) (36) where *pldA* (phospholipase) *H. pylori* showed similar adherence capability as that of

the wild type *in vitro* but failed to colonize in the stomach of mice *in vivo*. Similar findings were also observed in the study of *flaA H. pylori* mutant (37) and *ureB* mutant in the gnotobiotic piglet model (38). Dorrell *et al.* (36) proposed that the bacterial mutant could adhere to gastric epithelium causing "transient adhesion" for a few days but was unable to further evade and colonize in the stomach of animals. Like the *pldA H. pylori* mutant (36), it is believed that the *hsp20*-isogenic mutant have had a "transient adhesion" in the gastric stomach of BALB/c mice. It is also crucial to note that, using RT-PCR, the major adhesins (e.g. OipA, HopZ and SabA) examined in this study were all functional (i.e. under "ON" status) in both wild type and the isogenic mutant *H. pylori*. Thus, our findings may imply that *hsp20*-isogenic *H. pylori* could adhere to the gastric mucus probably through one or more of these multiple adhesins [e.g. OipA, HopZ, SabA and Le (X)] for a short period of time but then loses its ability to colonize on the gastric epithelium of the animals. In comparison with the adherence property shown *in vitro*, the role of HSP20 in bacterial colonization is more crucial *in vivo*. It is therefore appropriate to imply that HSP20 is mainly responsible in assisting in the colonization of bacteria *in vivo*, apart from playing a minor role in adhesion.

In an attempt to study the interaction between HSP20 and other proteins in *H. pylori*, co-immunoprecipitation (CO-IP) using antibody against rHSP20 showed that a single protein, CagA was pulled down from the protein extract of the wild type but not in the isogenic *H. pylori* (Fig 4). This highlights the potential role of HSP20 in interacting with CagA in *H. pylori*. The absence of this interaction in *hsp20*-isogenic mutant, CagA-negative *H. pylori* and non-*H. pylori* (*E. coli*) (serving as negative controls) further illustrates that the interaction between HSP20 and CagA is novel and unique.

CagA, being a major virulence factor of *H. pylori* (39), its presence in *H. pylori* is important for the pathogenesis of bacterial infection (40; 41). Using RT-PCR analysis of *cagA* in both *H. pylori* strains (wild type and the isogenic mutant) revealed that the expression level of *cagA* in the isogenic *H. pylori* is similar to that of the wild type regardless of the ages of *H. pylori* cultures examined (Fig 5A). This may imply that HSP20 is not related to the expression of *cagA* gene.

The surface presentation of CagA has been reported to be important for the pathogenesis of *H. pylori* infection (40; 41). Western blotting was used to examine the presence of CagA protein in collaborating with HSP20, a surface localized protein (18) in different sub-cellular fractions of *H. pylori*. The absence of CagA in AGE fraction (acid glycine extract comprising membrane and membrane associated proteins) and a comparatively lower abundance of CagA protein in OMP fraction (outer membrane proteins) of the isogenic *H. pylori* (Fig 6) could be correlated to the disruption of HSP20. Interestingly, with the addition of rHSP20 into the isogenic *H. pylori* culture, CagA emerged in all these 3 fractions [total proteins (TP), AGE & OMP] of the isogenic mutant with similar intensity as that of the wild type. This further strengthens the relationship between HSP20 and the presence of CagA in *H. pylori*. The close relationship between HSP20 and the presence of CagA in the surface fractions (AGE and OMP) signifies that HSP20 is important in collaborating with the presentation of CagA in *H. pylori*. It is postulated that the disrupted HSP20 in the isogenic *H. pylori* had resulted in the reduction of CagA in OMP fraction but was compensated by the addition of rHSP20 during culturing. Furthermore, the absence of CagA in the AGE fraction of the isogenic mutant while its restoration with the addition of rHSP20 may imply that HSP20 could be required for the

stabilization of CagA under low pH conditions, similar to the acidic environment in the stomach of the host (as AGE was prepared at pH 2.2).

As a major antigen in *H. pylori*, CagA could induce high antibody titer in humans and the infected animals during bacterial infection (42; 43). Hence, the potential collaboration between HSP20 and the presentation of CagA on *H. pylori* was further tested using antibody against CagA in *H. pylori* infected mice. The antibody level against CagA in *hsp20*-isogenic *H. pylori* infected mice was found to be significantly lower ($p<0.05$) than that of the wild type (Fig 7A). This may explain the good correlation between low abundance of CagA detected in the surface protein fraction of the isogenic *H. pylori* and the low antibody response against CagA in the isogenic mutant infected mice. It is possible that the disrupted HSP20 in the isogenic mutant could have led to the ineffective presentation of CagA or partial CagA as antigen on the cell surface of *H. pylori* resulting in the reduction of antibody induced during *H. pylori* infection. This may further support the probable involvement of HSP20 in the presentation and stabilization of CagA on the cell surface of *H. pylori*. As a heat shock protein, HSP20 could probably function as an assisting factor in "delivering" and/or "presenting" CagA onto the cell surface of *H. pylori*. It is therefore proposed that HSP20 may serve as a "chaperon" for the virulence factor, CagA in *H. pylori*.

Our findings from this study suggest that HSP20 is an important factor assisting in the colonization of *H. pylori* in mice. Its role in the bacterial colonization is independent of the *H. pylori* adhesins like OipA, HopZ and SabA.

With the significant contribution of CagA to the pathogenecity of *H. pylori* infection (40; 41), the strong association between HSP20 and CagA represents an indirect but critical

role in the bacterial pathogenesis of *H. pylori* infection. This study emphasizes the different role of HSP20 in *H. pylori* as compared to that of the HslV, a component of ATP-dependent protease in *E. coli* (44). From the identification of interaction between HSP20 and CagA to the possible chaperonic role of HSP20 through the surface presentation of CagA, it further addresses the importance of HSP20 in *H. pylori* infection. This study on HSP20 in *H. pylori* opens up an insight in studying the bacterial-host interaction and understanding on the development of effective anti-*H. pylori* therapeutics against *H. pylori* infection.

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Table 1. Primers used in this study

Name of primer	Gene	Sequences (5' – 3')	Length (bp)	Method(s)	Ref.
KO1	HP0513	CGGGATCCATGAACGGACATTTTATCGGTT (BamHI)	2700	PCR	(20)
KO2	HP0515	AACTGCAGCCATTCTTTACTGAAATCCACC (PstI)			
KO3	HP0515	ACGCGTCGACCGCAAAGATAAGTATTTACGC (SalI)	2549	PCR	(20)
KO4	HP0517	TCCGGGCCCTCAATCCCTATTCCTTCTATGGA (ApaI)			
HSP20-F	HP0515	CGGGATCCATGTTTGAAGCGACGACGATTTTAGGC	543	PCR	(20)
HSP20-R	HP0515	CGGGATCCTTAAAGCTCCAAAATTTTAATATTCGTG			
Km-F	*aphA*	CGGGATCCGATAAACCCAGCGAACCATTTGAG	1340	PCR	(45)
Km-R	*aphA*	CGGGATCCAAGCTTTTTAGACATCTAAATCTAGGT			
T7	-	GTAATACGACTCACTATAGGGC	-	PCR	*
16s rRNA-F	16s rRNA	GGAGGATGAAGGTTTTAGGATTG	390	RT-PCR	(29)
16s rRNA-R	16s rRNA	TCGTTTAGGGCGTGGACT			
oipA-FS	HP0638	CAA GCG CTT AAC AGA TAG GC	-	PCR & sequencing	(27)
oipA-RS	HP0638	AAG GCG TTT TCT GCT GAA GC			
hopZ-FS	HP0009	GCC TGA TAT GGG TGG CAT GGG	-	PCR & sequencing	(27)
hopZ-RS	HP0009	ATT TGA TAG CCC GCG CTG AT			
sabA-FS	HP0725	TTT TTG TCA GCT ACG CGT TC	-	PCR & sequencing	(26)
sabA-RS	HP0725	ACC GAA GTG ATA ACG GCT TG			
babA2-F	*babA2*	AATCCAAAAAGGAGAAAAAGTATGAAA	810	PCR	(28)
babA2-R	*babA2*	TGTTAGTGATTTCGGTGTAGGACA			
oipA-F	HP0638	ATGAGCTCAGCTTTGGGTATAA	457	RT-PCR	(20)
oipA-R	HP0638	GCGATCAATATCGTATTCATCA			
hopZ-F	HP0009	ACTACTACTACTACTAATGACG	611	RT-PCR	(20)
hopZ-R	HP0009	AATCCTTAAGGCTGCCTCTAAA			
sabA-F	HP0725	ATCCACTAATTACCCAACGCAAT	643	RT-PCR	(20)
sabA-R	HP0725	GTCGTTATAGGCGGTTACGATT			
cagA-F	HP0547	GGAACGCCATATGATGACTAACGAAACCATTG	852	RT-PCR	(20)
cagA-R	HP0547	CGCGGATCCTTAATCAATGTCAGCGACTCCC			

Restriction enzyme sites are underlined. * Stratagene, CA, USA.

Table 2. Comparison of adherence ability of *hsp20*-isogenic and wild type *H. pylori*

Assays	Bacteria:cell ratio	Adherence to KATO III (%)	Adherence to AGS (%)
		Mutant / WT	Mutant /WT
ELISA	50:1	83.2 ± 1.9	88.3 ± 1.0
	100:1	86.5 ± 0.9	91.6 ± 0.8
	200:1	74.9 ± 1.1	86.3 ± 1.2
Flow Cytometry	50:1	86.7 ± 0.5	86.4 ± 0.5
	100:1	85.6 ± 0.4	83.9 ± 0.6
	200:1	91.6 ± 0.4	88.0 ± 0.5

Table 3. Analysis of *H. pylori* colonization in mice

H. pylori inoculated	Assays	Rate of *H. pylori* detected			Total
		2 weeks	4 weeks	8 weeks	
Wild Type	Microbiological	5/5	5/5	5/5	15/15 (100%)
	Histological	3/5	3/5	3/5	15/15 (100%)
	RT-PCR	5/5	5/5	5/5	15/15 (100%)
hsp20-isogenic mutant	Microbiological	0/5	0/5	0/5	0/15 (0%)
	Histological	0/5	0/5	0/5	0/15 (0%)
	RT-PCR	0/5	0/5	0/5	0/15 (0%)

Table 4. Gene status of *H. pylori* adhesins

Gene	Strains	Partial sequences	Number of CT repeats	Gene status
***oipA* (HP0638)**	SS1 (WT)	ATGAAAAAAGCTCTCTTACTAACTCTCTCTCTCTCTCGTTTTGG M K K A L L L T L S L S F W	6	On
	hsp20-isogenic mutant	ATGAAAAAAGCTCTCTTACTAACTCTCTCTCTCTCTCGTTTTGG M K K A L L L T L S L S F W	6	On
***hopZ* (HP0009)**	SS1 (WT)	ATGAAAAAAACCCTTTTACTCTCTCTCTCTCTCGCTTCATCG M K K T L L L S L S L A S S	7	On
	hsp20-isogenic mutant	ATGAAAAAAACCCTTTTACTCTCTCTCTCTCTCGCTTCATCG M K K T L L L S L S L A S S	7	On
***sabA* (HP0725)**	SS1 (WT)	ATGAAAAAGACAATTCTGCTCTCTCTCTCTCTCGCTTCATCG M K K T I L L S L S L A S S	7	On
	hsp20-isogenic mutant	ATGAAAAAGACAATTCTGCTCTCTCTCTCTCTCGCTTCATCG M K K T I L L S L S L A S S	7	On

The CT dinucleotide repeats were highlighted underline.

Figure legend

Fig 1. Schematic construction of *hsp20::aphA* gene-targeting vector

A, the location of genes in genome of *H. pylori* 26695; four primers (KO1 – 4) were designed based on the known sequences; **B**, two flanking DNA fragments (5' & 3') were amplified using *H. pylori* SS1 genomic DNA as template with the respective restriction sites on their flanking ends according to multiple cloning sties of pBluescript SK (+); **C**, the insertion of the two flanking fragments (5' & 3') and *aphA* gene into pBluescript SK (+); the full length of targeting vector (pBS-5'-*aphA*-3') is 9589bp.

Fig 2. Detection of antibody in *H. pylori* inoculated mice

A, total antibody against *H. pylori* detected in both *H. pylori* inoculated mice; **B**, antibody against HSP20 detected in mice. WT, wild type *H. pylori* inoculated mice; MUTANT, *hsp20*-isogenic *H. pylori* inoculated mice; NEGATIVE, negative control mice fed with BHI broth alone. *The differences between wild type and mutated *H. pylori* inoculated mice were compared and statistically significant ($p<=0.01$).

Fig 3. RT-PCR analyses of adhesin genes of *H. pylori*

A, RT-PCR analysis of three adhesin genes of *H. pylori*; lanes 1 & 2, *oipA* gene fragment (457 bp); lanes 3 & 4, *hopZ* gene fragment (611 bp); lanes 5 & 6, *sabA* gene fragment (643 bp); lanes 1, 3 & 5, RT-PCR using wild type *H. pylori* as template; lanes 2, 4 & 6, RT-PCR using *hsp20*-isogenic *H. pylori* as template. M1, 1 kb DNA ladder; M2, 100 based pair DNA ladder. **B,** RT-PCR analysis of 16s rRNA gene fragment (390 bp); WT, RT-PCR using wild type *H. pylori* as template; Mu, RT-PCR using *hsp20*-isogenic *H. pylori* as template; M, 100 base pair DNA ladder.

Fig 4. CO-IP analysis and protein identification

A, SDS-PAGE (12%) of CO-IP with antibody against rHSP20; lane 1, CO-IP of *E. coli* cells with rHSP20 Ab (negative control); lane 2, CO-IP of *H. pylori* SS1 cells with pre-immune rabbit serum (internal negative control); lane 3, CO-IP of *hsp20*-isogenic mutant *H. pylori* proteins with rHSP20 Ab; lane 4, CO-IP of wild type *H. pylori* proteins with rHSP20 Ab. M, prestained Precision Protein Standards (Biorad). The isolated protein was indicated as arrow. **B**, the amino acids sequence of CagA protein identified (acc.

P55746 in NCBI) by MS from CO-IP; the matched peptides were in gray color and underlined; 11 out of 34 peptides matched with 32% identity. **C & D**, Western blots probed with antibody against rCagA (C) and antibody against rHSP20 (D) after CO-IP reaction, lane 1, CO-IP of wild type *H. pylori*; lane 2, CO-IP of CagA-negative *H. pylori*; lane 3, CO-IP of *hsp20*-isogenic *H. pylori* mutant, M, prestained Precision Protein Standards (Biorad).

Fig 5. *cagA* expression in *H. pylori* analyzed by RT-PCR

A, amplification of *cagA* gene fragment (852 bp) in *H. pylori*; **B**, amplification of 16s rRNA gene fragment (390 bp). M1, 100 bp DNA ladder; M2, 1 kb DNA ladder (Biolabs). Lanes 1 & 2, amplification based on the 3rd and 4th day culture of wild type *H. pylori*; lanes 3 & 4, amplification based on the 3rd and 4th day culture of *hsp20*-isogenic *H. pylori.*

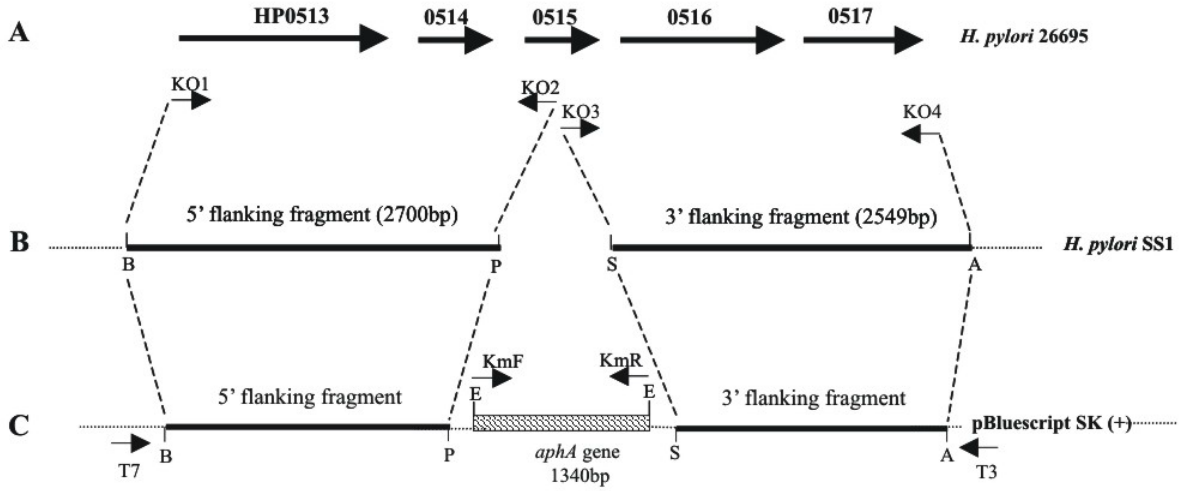
Fig 6. Presence of CagA in different *H. pylori* sub-cellular fractions

A, different *H. pylori* cultures grown in BHI broth; **B**, different *H. pylori* cultures grown in BHI broth supplemented with 0.5 μg/ml rHSP20. The amount of 10 μg protein was loaded per lane in 10% SDS-PAGE. Lanes 1, 4 & 7, total protein (TP) extract of *H. pylori*; lanes 2, 5 & 8, acid glycine extract (AGE) of *H. pylori*, lanes 3, 6 & 9, outer membrane protein (OMP) extract of *H. pylori*. Lanes 1 –3, protein extracts of wild type *H. pylori*, lanes 4 – 6, protein extracts of CagA-negative *H. pylori*; lanes 7 – 9, protein extracts of *hsp20*-isogenic *H. pylori* mutant. M, prestained Precision Protein Standards (Biorad). The Western blot was carried out using antibody against rCagA or antibody against HSP60.

Fig 7. Antibody against CagA and Lewis antigens in *H. pylori* infected mice

A, antibody against CagA detected in *H. pylori* infected mice. *The differences between wild type and *hsp20*- isogenic *H. pylori* inoculated mice were compared and statistically significant ($p<0.05$); **B**, antibody against Le (X) antigen in *H. pylori* infected mice; **C**, antibody against Le (Y) antigen in *H. pylori* infected mice. WT, wild type *H. pylori* inoculated mice; MUTANT, *hsp20*-isogenic *H. pylori* inoculated mice; NEGATIVE, negative control mice fed with BHI broth alone.

Fig 1. Schematic construction of *hsp20::aphA* gene-targeting vector



Restriction sites: B: BamHI; P: PstI; S: SalI; A: ApaI and E: EcoRI.
HP0515 is the target gene, *hsp20*.

Fig 2. Detection of antibody in *H. pylori* inoculated mice

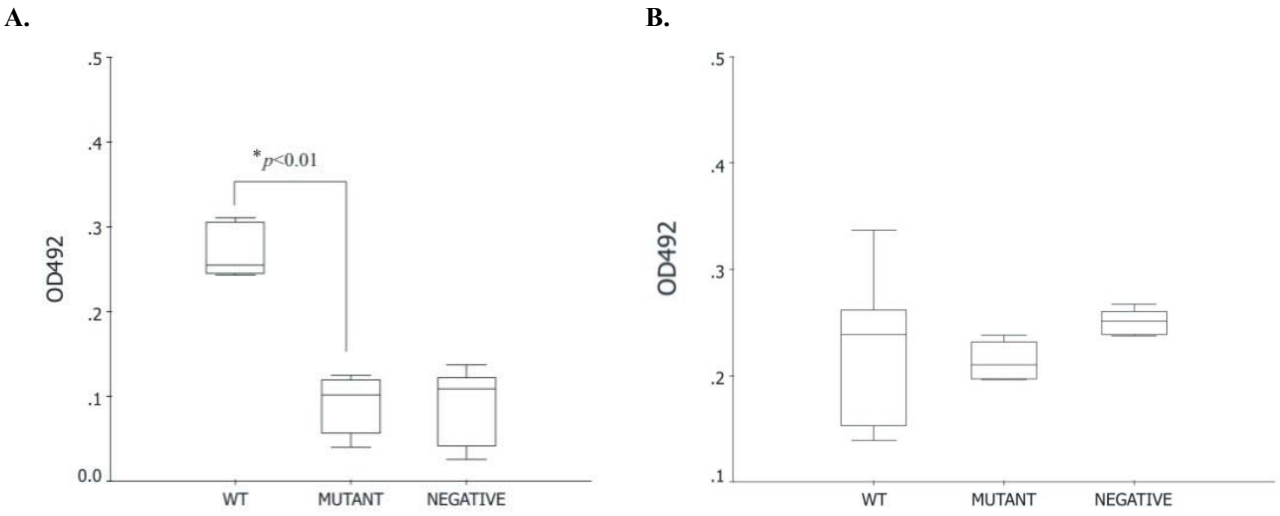


Fig 3. RT-PCR analyses of adhesin genes of *H. pylori*

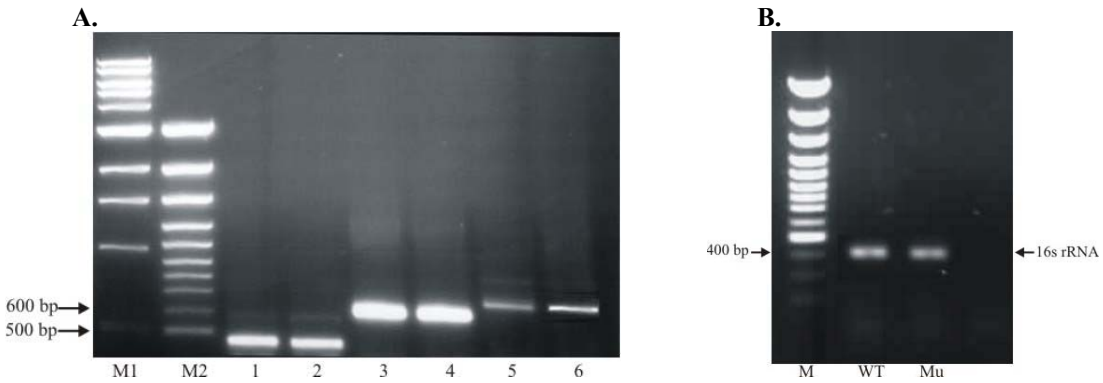
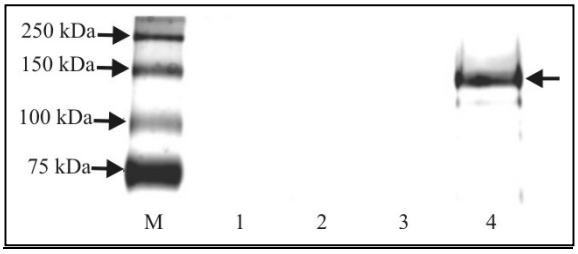


Fig 4. CO-IP analysis and protein identification

A.



B.

MTNETIDQQPQTEAAFNPQQFINNLQVAFLKVDNAVASYDPDQKPIVDKNDRDNRQAFEGISQLREEYSNKAIKNPTKKNQYFSDFINKSNDLINKDNLIVVESSTKSFQ
KFGDQRYRIFTSWVSHQNDPSKINTRCIRNFMEHTIQPPIPDDKEKAEFLKSAKQSFAGIIIGNQIRTDQKFMGVFDESLKERQEAEKNGGPTGGDWLDIFLSFIFDKKQ
SSDVKEAINQEPLPHVQPDIATSTTHIQGLPPESRDLLDERGNFSKFTLGDMEMLDVEGVADMDPNYKFNQLLIHNNTLSSVLMGSHDGIEPEKVSLLYAGNGGFGAKHD
WNATVGYKDQQGNNVATIINVHMKNGSGLVIAGGEKGINNPSFYLYKEDQLTGSQRALSQEEIQNKIDFMEFLAQNNAKLDSLSEKEKEKFRNEIKDFQKDSKPYLDALG
NDRIAFVSKKDPKHSALITEFNKGDLSYTLKVMGKKQIKALDREKNVTLQGNLKHDGVMFVNYSNFKYTNASKSPNKGVGVTNGVSHLEAGFSKVAVFNLPNLNNLAITS
VVRRDLEDKLIAKGLSPQEANKLVKDFLSSNKELVGKALNFNKAVAEAKNTGNYDEVKRAQKDLEKSLKKREHLEKGDVAKNLESKSGNKNKMEAKAQANSQKDEIFALI
NKEANRDARAIAYAQNLKGIKRELSDKLENINKDLKDFSKSFDGFKNGKNKDFSKAEETLKALKGSVKDLGINPEWISKVENLNAALNEFKNGKNKDFSKVTQAKSDQEN
SIKDVIINQKITDKVDELNQAVSVAKIACDFSGVEQALADLKNFSKEQLAQQAQKNESFNVGKSEIYQSVKNGVNGTLVGNGLSGIEATALAKNFSDIKKELNEKFKNFN
NNNNNGLKNGGEPIYAQVNKKKTGQVASPEEPIYAQVAKKVTKKIDQLNQAATSGFGGVGQAGFPLKRHDKVEDLSKVGRSVSPEPIYATIDDLGGSFPLKRHDKVDDLS
KVGLSRNQELTQKIDNLSQAVSEAKAGFFGNLEQTIDKLKDFTKNNPVNLWAESAKKVPASLSAKLDNYATNSHTRINSNIQNGAINEKATGTERQKNPEWLKLVNDKIV
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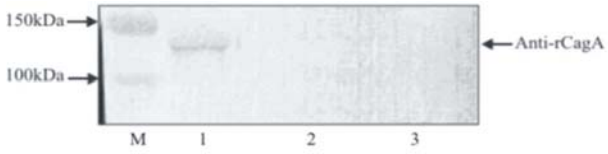
C.



D.

Fig 5. *cagA* expression in *H. pylori* analyzed by RT-PCR

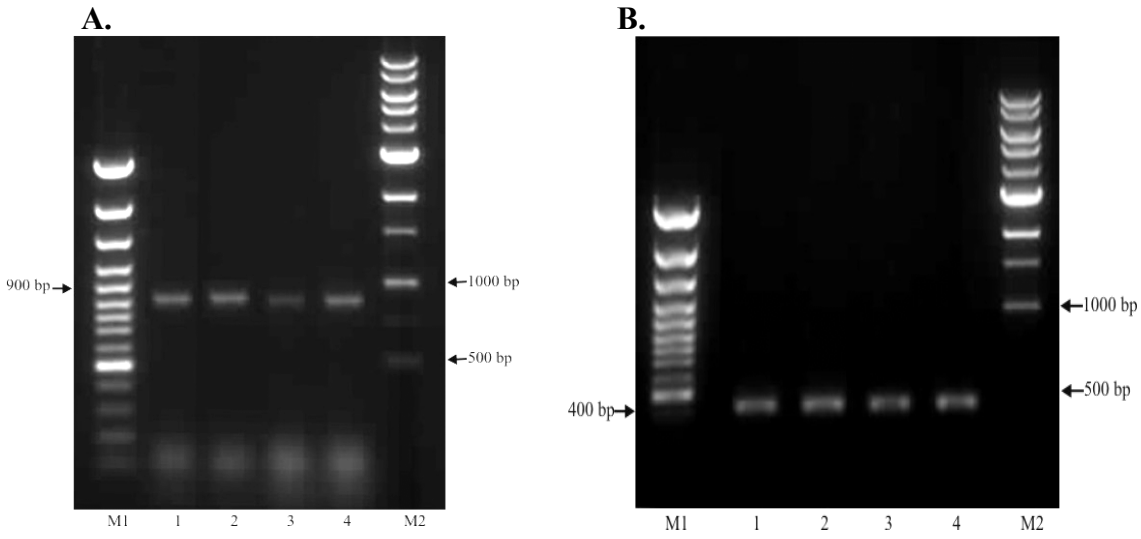


Fig 6. Presence of CagA in different *H. pylori* sub-cellular fractions

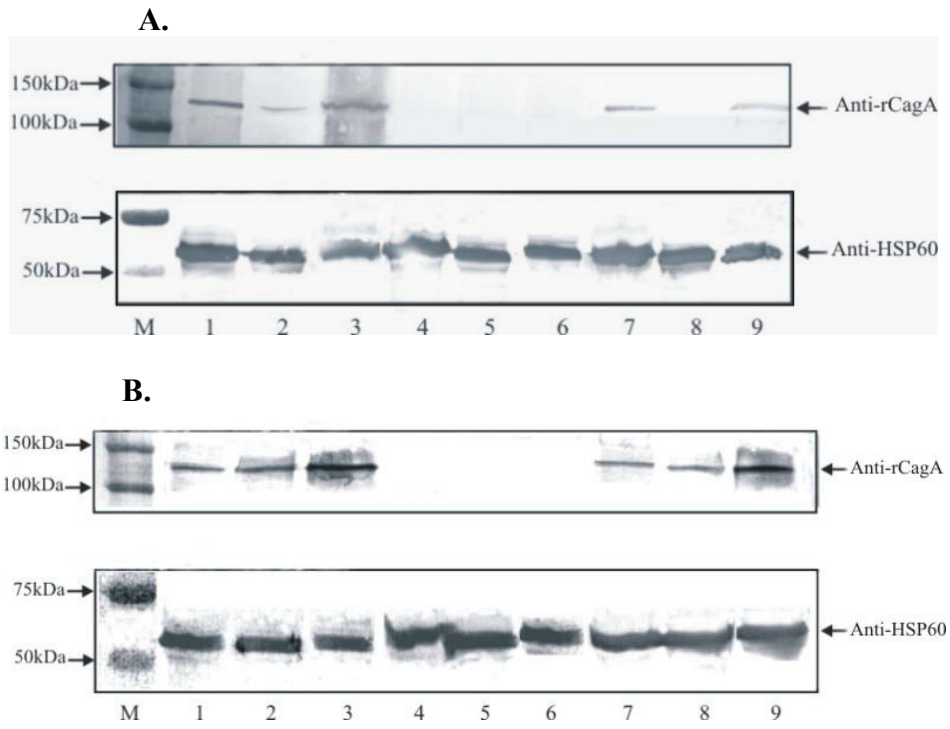
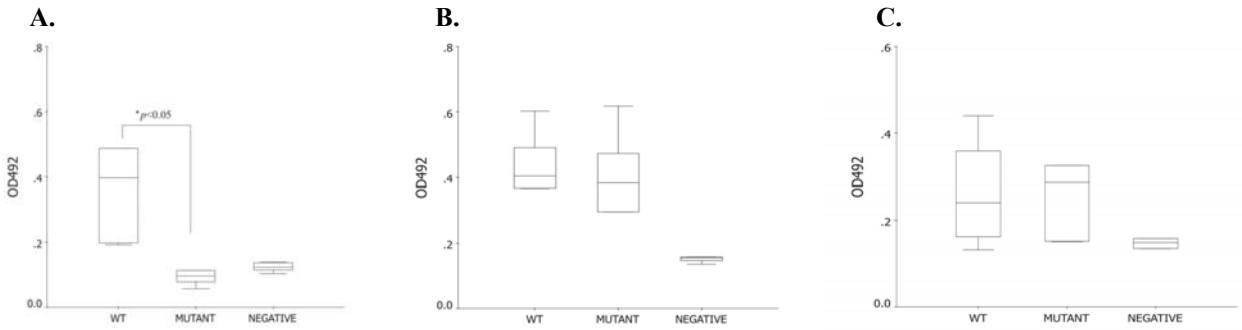


Fig 7. Antibody against CagA and Lewis antigens in *H. pylori* infected mice



Heat Shock Protein 20 of *Helicobacter pylori* – A novel epidemiological and gastroduodenal disease differentiating marker

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Key words: *Helicobacter pylori*; HSP20; substitution; epidemiological marker, gastroduodenal diseases.

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Abstract

Background: Notwithstanding the prevalence of *H. pylori* infection in different regions of the world, no disease epidemiological marker for *H. pylori* infection has been established.

Aims: Using a newly identified surface localized heat shock protein 20 (HSP20) that was shown to be involved in colonization of *H. pylori*, the relevance of the genetic structure of *hsp20* (HP0515) to *H. pylori* infection was explored.

Methods: *hsp20* gene sequences of 225 *H. pylori* isolates with different gastroduodenal disease outcome from 10 countries and 2 standard strains (26695 and J99) were analyzed using phylogenetic tools. The 3-D protein structure of HSP20 was predicted based on molecular modeling.

Results: *hsp20* gene was found to be conserved in all *H. pylori* strains studied. The phylogram generated from *hsp20* DNA sequences demonstrated two geographical clusters: Asian and non-Asian groupings. Interestingly, the distinctive substitutions at 14^{th} – 16^{th} amino acid residues of HSP20 exhibited strong association with the two geographical groupings: M-G-G cluster with Asian origin while F-D-N cluster with the non-Asian origin. Uniquely, the M-G-G and F-D-N substitutions were also shown to be associated with PUD (peptic ulcer disease) and NUD (non-ulcer dyspepsia), respectively.

Conclusion: HSP20 with unique substitutions at 14^{th} – 16^{th} may represent the adaptation of *H. pylori* to different environments or reflect the random genetic drift without effects on phenotype thereby representing it as a potential epidemiological marker. It is also postulated that substitutions M-G-G has its origin in Asia. More interestingly, the 3-amino-acid substitutions have shown the capability to differentiate NUD from PUD.

Introduction

Helicobacter pylori is a microaerophilic, gram-negative spiral. This human gastric bacterial pathogen has been associated with gastritis, peptic ulcer and gastric cancer in patients across continents, races and age groups.[1] The variation of infections may be contributed by the high genetic diversity of bacterial genomes of *H. pylori* isolates[2] or a combination of host and pathogens.[3] With the availability of two genomic DNA sequences of *H. pylori* 26695 and J99,[4][5] the genetic diversity of bacterial genome has been viewed in the genome wide manner. Comparison between these two genomes shows that there are about 6 – 7% of the annotated genes that are strain specific but are absent from each other with no identifiable homologue in the databases.[4]

Housekeeping genes (*atpA & D, efp, mutY, ppa, trpc, urel, yphC, recA, glnA, scoB*), virulence associated genes (*cagA, vacA, iceA*) and transposable elements (IS605 & IS608) have been sequenced for use to analyze the genetic diversity between strains.[6][7][8] Sequencing studies showed that it was extremely rare for an orthologous gene from different *H. pylori* strains to have the same sequence.[9][10] The phylogenetic studies based on different genes (e.g. housekeeping genes, virulence factor genes & transposable elements)[6][7][8] showed the existence of recombination and geographical-origin-based clustering (Western and Asian) among *H. pylori* isolates.

Epidemiological study has shown that *H. pylori* infection is more prevalent in developing countries, in particular East Asia.[11] Furthermore, the more severe gastroduodenal cases like peptic ulcer diseases and gastric cancer were observed mainly in Asian populations.[12][13] Besides the host genotypes and environmental factors, virulence of *H. pylori* strains determines bacterial pathogenicity that might be related to

the severity of gastroduodenal diseases.[3] However, no evident genetic marker has been established to distinguish *H. pylori* isolates with respect to the disease status due to *H. pylori* infection. Therefore, differentiating *H. pylori* isolates from Asian or Western countries would benefit the study of *H. pylori* and its relationship with clinical outcome in patients.

HSP20 is a newly identified surface localized protein in *H. pylori*.[14] Our recent study showed that HSP20 is involved in the colonization of *H. pylori* in mice (manuscript submitted). Its cooperative role in the initial stage of *H. pylori* infection implies its importance in the bacteria-host interactions. Due to the significant contribution of HSP20 in the process of *H. pylori* infection, it is therefore interesting to understand the genetic structure of HSP20 in differentiating various *H. pylori* infections. Furthermore, the relevance of *hsp20* gene for use as an epidemiological marker of *H. pylori* was also explored.

Material and Methods

H. pylori Strains

A total of 225 *H. pylori* isolates obtained from different geographical regions and races were included in this study (Table 1). Among these, 103 strains were isolated from Singapore and 122 strains were from 9 different countries. All *H. pylori* isolates were cultured on chocolates blood agar plates supplemented with antibiotics.[15] The plates were incubated at 37°C in an atmosphere of 5% CO_2 in a humidified incubator (Forma Scientific, Marietta, OH, USA).

DNA isolation, amplification, and sequencing

Genomic DNAs of *H. pylori* isolates were extracted according to the method as described by Hua *et al*.[16] A pair of primers corresponding to 50bp upstream and downstream of *hsp20* (HP0515) of *H. pylori* 26695 genomic sequences was used.[5] The forward primer was: 5'> CGGAATTCAGATTGAAGTCAAGC <3' while the reverse primer was: 5'> CGGGATCCTGCCCAATGATGTATT <3'. PCR amplification was carried out by an initial denaturation at 94°C for 5 minutes followed by 94°C, 30 seconds; 50°C, 30 seconds; 72°C, 30 seconds for 30 cycles with additional extension at 72°C for 5 minutes. The PCR amplified 643bp gene fragments were purified by PCR product purification kit (Qiagen, Hilden, Germany) and sequenced using BigDye ™ Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, Wellesley, MA, USA) in ABI 100 model 377 DNA sequencer (Perkin Elmer, Wellesley, MA, USA).

Sequences alignment and comparison

hsp20 gene sequences from 225 isolates and the two known genomic DNA sequences strains (in 26695, Genebank accession no: AE000566; in J99, Genebank accession no: AE001480)[4][5] were aligned and the corresponding amino acid sequences were deduced. Multiple alignment of sequences was conducted using ClustalX version 1.81.[17] The GC content, polymorphic sites, percentages of the mean differences between pairs of strains at synonymous nucleotide positions (K_S) and nonsynonymous nucleotide positions (K_A) were calculated with DNASP version 3.5.[18]

Phylogenetic analysis

PHYLIP (the PHYLogeny Inference Package) Version 3.6 was used to conduct phylogenetic analysis[19] based on DNA sequences of 227 strains of *H. pylori*. The

maximum likelihood (ML) algorithm was chosen and performed following the procedures proposed by Baxevanis *et al.*[20] A bootstrap analysis (100 replicates) was performed to evaluate the topology of the phylogenetic trees. The nucleotide divergence between groups was estimated by using Jukes-Cantor methods in DNASP 3.5.[18]

Protein structure prediction

SWISS-MODEL[21] Server available at http://www.expasy.org/swissmod/ was used to predict protein structure of HSP20. The amino acid sequence of HP0515 of *H. pylori* 26695 was used as a query for finding homologous templates from ExPDB database. The two best-scored templates chosen for modeling are ATP-dependent protease HslV of *Haemophilus influenzae* showing 57% identity (PDB accession code: 1kyiL; Genebank accession no: U32731)[22] and HslV of *Escherichia coli* showing 49% identity (PDB accession code: 1e94A; Genebank accession no: AE000467).[23] The predicted protein structure was evaluated by WHATCHECK program[24] that suggested the stereochemistry and energetic parameters of the model was acceptable.

Structure comparison of substitutions at 14^{th} – 16^{th} amino acid residues

The amino acid sequences of *hsp20* that represent seven major types of substitutions respectively were modeled using SWISS-MODEL Sever. These strains were *H. pylori* J99 whose substitutions is F-D-N at 14^{th} –16^{th}, *H. pylori* 26695 for L-N-H, Singapore isolates RH54 for M-G-G, Swedish isolates 58 for M-E-G, Japanese isolates 1107 for I-G-G, Sweden isolates 24 for M-R-G and Swedish isolates 88 for F-N-H. Seven types of amino acid substitutions were examined based on its position in the 3-D structure.

Results

Nucleic acid sequence analysis

The nucleic acid sequences of *hsp20* of all 227 strains showed an open reading frame of 540bp with no deletion and insertion. However, there were a total of 219 polymorphic sites scattered in the whole gene fragment, showing high level of synonymous sequence variations. Most of the nucleotide substitutions (57.99%) were at the third codon position. The (G+C)% content of all *hsp20* sequences analyzed ranges from 41.25% to 44.57% with an average of 43.12%. The percentage of the differences between pairs of strains at synonymous nucleotide positions (*Ks*) was 15.9% and 1.25% at non-synonymous positions (*Ka*). The ratio of *Ks/Ka* was 12.65.

Amino acid sequence analysis

The corresponding amino acid sequences deduced from nucleic acid sequences showed a total number of 51 substitutions. Of these, 79% of the amino acid substitutions belongs to the same polarity group, e.g. from polar to polar or from hydrophobic to hydrophobic. The remaining 21% was substituted between different groups, e.g. switching between polar and hydrophobic.

There are seven types of substitutions observed at 14^{th} – 16^{th} amino acid residues. The substitutions corresponding to positions 14^{th}-15^{th}-16^{th} were: M-G-G (~76%), M-E-G (~4%), M-R-G (~0.4%) and I-G-G (~3%) constituting the M-G-G cluster while the F-D-N cluster comprises F-D-N (~13.6%), L-N-H (~2.2%) and F-N-H (~0.8%). The substitutions were more diverse among the non-Asian group (M-G-G, F-D-N, L-N-H, M-E-G, F-N-H & M-R-G) than the Asian group (M-G-G, I-G-G, M-E-G & F-D-N). Interestingly, substitution M-G-G predominates among the Asian group (91%, 143/158)

while the F-D-N substitution is found more frequently in *H. pylori* isolates obtained from non-Asian countries (39%, 27/69) (Tables 1). Among the seven observed substitutions, M-G-G is the most prevalent type in all tested isolates (76%, 172/227).

Interestingly, substitutions at 14^{th} – 16^{th} amino acid residues (Table 2) show that significantly more patients with PUD (peptic ulcer disease) harbored *H. pylori* isolates with M-G-G substitution cluster (M-G-G, M-E-G, M-R-G and I-G-G) while those patients with NUD (non-ulcer dyspepsia) possessed *H. pylori* strains with F-D-N substitution cluster (F-D-N, L-N-H and F-N-H). Based on the diseases outcome available for 195 out of 227 *H. pylori* trains in this study, the odds ratio (OR) of M-G-G cluster for PUD was calculated.[25] It was shown that M-G-G has an OR of 4.27 in its association with PUD as compared to F-D-N that is positively associated with NUD (Table 2).

Phylogenetic analysis

The dendrogram as shown in Fig 1 was generated based on the analyses of nucleic acid sequences of *hsp20* from 225 tested strains and the 2 strains (26695 & J99) with known genomic DNA sequences.[4][5] Using PHYLIP and ML algorithm, the inferred taxonomic distance between different *H. pylori* strains was arrived as illustrated in Fig 1. Two major clusters (A & B) were observed: a larger group A and a smaller group B. The isolates in group A shows high similarity in its DNA sequences and further bifurcates into two subgroups, namely A_1 and A_2. Interestingly, subgroup A_1 and group B were mainly from Asian and non-Asian origins, respectively. However, subgroup A_2 comprises a mix of *H. pylori* isolates of Asian and non-Asian origins. In group A_1 (n=119), all the strains except for CR10498 (Costa Rica), SJM1 and SJM14 (Peruvian) and Cau1026 (an isolate from an European visitor in Singapore) are entirely from Asian countries. There

were 34 Asian strains and 36 non-Asian strains that made up group A_2 (n=70). The remainder (n=35) including four Singapore strains (Sin1059, 541, 1134 & 1024, all were isolates of Malay ethnic origin) constituted group B. There were 3 strains (J1186, HK77 and SJM19) which were not aligned to any of the groups. The percentage of distribution shows that Asian isolates are present in 96.64% (group A_1), 48.57% (group A_2) and 11.43% (group B) while non-Asian isolates are present in 3.36% (group A_1), 51.43% (group A_2) and 88.57% (group B).

The observation was further confirmed by the estimated DNA divergence as shown in Table 3. The nucleotide divergence (*D*) within group A_1 (2.43%), A_2 (3.38%) or group B (3.69 %) was relatively lower than the *D* value between groups. The divergence between group A_1 and B (6.03 %) was the highest followed by A_2 and B (5.29 %) or A_1 and A_2 (4.34%). The value of estimated *Ks* between different groups (Table 4) from high to low was in the order of A_1 vs. B (0.212); A_2 vs. B (0.190) and A_1 vs. A_2 (0.185). Furthermore, it is noted that the divergence differences between different *hsp20* gene groupings were significantly lower when compared with the divergence between *vacA* alleles m1 and m2 (24.9%) or the *Ks* between m1 and m2 (0.46) as reported by Atherton *et al.* (1999).[26]

Similarly, interesting findings were observed in regards to the substitutions at 14^{th} – 16^{th} amino acid residues. The predominant substitution is M-G-G that comprises 172/227 isolates (Table 1). Of these, 109 and 61 isolates with M-G-G substitution are located in group A_1 and A_2, respectively (Table 4). It shows that group A_1 is predominately the M-G-G substitution with Asian origin while group A_2 comprises a mix of M-G-G substitution of Asian and non-Asian origins. The other substitutions, I-G-G (n= 7) is only

found in Asian isolates and strictly distributed in group A_1; M-E-G (n=9) substitution are distributed in both group A_1 and A_2 while the substitution M-R-G with only a single strain of Swe24 is located in group A_2. In contrast, F-D-N substitution was found in 31/227 isolates (Table 1). F-D-N is the prevalent substitution type in group B (28/35) (Table 4). It is notable that except two F-D-N substitution (Aus3 and NCTC11637) clustered in group A_2, the rest of F-D-N substitution cluster (F-D-N, L-N-H and F-N-H) are located in the group B including four Singapore isolates (Sin1059, 541, 1134 & 1024, all were isolates of Malay ethnic origin).

Protein structure prediction

The predicted HSP20 protein structure shows four α helixes, ten β sheets and 14 turns (Figs 2 & 3). The four α helixes, two at each side, flank the central core of ten β sheets that are concentrated in the middle. All the α helixes and β sheets are evenly distributed on both sides forming a symmetrical structure.

Structure comparison of substitutions at 14^{th} – 16^{th} amino acid residues

The amino acids of 14^{th} – 16^{th} are located at the end of the first β sheet that display on the surface of predicted HSP20 protein 3-D structure (Figs 2 & 3). Interestingly, based on the obtained 3-D structure, the seven different substitutions (M-G-G, I-G-G, M-E-G, M-R-G, F-D-N, L-N-H and F-N-H) do not affect the protein conformation in the 3-D model predicted.

The 3-amino-acid (14^{th} – 16^{th}) substitutions is unique in *H. pylori* HSP20 species which is absent in both HslV of *E. coli* and *H. influenzae* as shown in the alignment of three homologues in Fig 4.

Discussion

This study centers on the DNA sequence of *hsp20* (HP0515). *hsp20* is conserved with an open reading frame (ORF) of 540bp in all *H. pylori* isolates studied. This is different from *cagA*, *vacA* and outer membrane protein (HP0638) gene sequences where gene polymorphism has led to insertion or deletion of gene fragments among strains. Atherton *et al*.[28] have shown that *vacA* has at least two variable regions: s region (s1a, s1b, s1c & s2) and m region (m1, m1T & m2). Similarly, HP0638 shows two dichotomies that is strongly correlated with *cagA* and *vacA* status.[29] *cagA* gene was shown to be present in 60% - 70% of *H. pylori* strains[30] among the Western population but was found in 80% - 90% of *H. pylori* isolates in Asian population.[31]

Based on the nucleotide acid sequences of *hsp20*, the phylogram plotted demonstrated two major clusters of *H. pylori* strains (Asian & non-Asian groupings) according to the geographical demarcation (A & B, Fig 1). Our results on the geographical groupings agree with previous studies in which the analyses were based on the sequence of housekeeping genes e.g. *recA*, *atpD*, *glnA*, *scoB* or genotyping of virulence genes *cagA*, *vacA* and transposable elements IS605 and IS608.[6][7][8] [27] Compared with other genes of *H. pylori*, the percentage of divergence (*D, Ks*) and the ratio of *Ks/Ka* (Table 3) between the 3 different *hsp20* genetic groupings (A_1, A_2 and B) were significantly lower than that of *vacA* alleles m1 & m2[26] or housekeeping genes (*atpD*, *scoB*, *glnA* and *recA*)[27] as reported. This may indicate that different genes of *H. pylori* are under the control of different selection pressures against amino acid replacement.[32] The divergence ((*D, Ks, Ka*) and the ratio of *Ks/Ka* (Table 3) values in the 3 different genetic groupings show that *hsp20* sequences are stable and effective in discriminatively

distinguishing *H. pylori* strains from different geographical origins. The evolutionary variations of *H. pylori* based on the findings of *hsp20* phylogeny are comparable with that of housekeeping genes[6][7][8] and surface proteins (HP0638).[29]

Although HSP20 (HP0515) is a homologue of HslV in *Haemophilus influenzae*[22] and *E. coli*,[23] the 3-amino-acid substitutions at 14^{th} –16^{th} is unique in HSP20 as it is not present in the homologues of the other two bacterial species (Fig 4), implying that these 3-amino-acid residues could have acquired during the evolutionary process of *H. pylori* and may represent the adaptation of bacteria to different environments or reflect the random genetic drift without effects on phenotype. Hence, it further indicates the distinctiveness of *hsp20* and the 3-amino-acid substitutions.

It is interesting to note that the unique 3-amino-acid substitutions of all HSP20 amino acid sequences exhibited similar geographical affiliation as the *hsp20* DNA sequences. In the former, these two clusters can be divided into two broad groups: M-G-G and F-D-N clusters. The change of substitutions occurs with the transition of one or more nucleotide, e.g. in the case of M-G-G and I-G-G, the substitution from methionine (M) to isoleucine (I) would have resulted from the nucleotide transition of ATG [methionine (M)] to ATA or ATT [isoleucine (I)]; similarly, the other substitutions M-G-G to M-E-G and M-R-G occurred with a transition of one nucleotide for the middle glycine G, (GGG) to glutamate E, (GAG) or arginine R, (AGG) (Fig 5). These are considered as M-G-G substitution cluster. The other substitution cluster comprising of F-D-N, F-N-H & L-N-H is termed as F-D-N cluster. The nucleotide transition in F-D-N cluster occurs in more than one single "mutation" from M-G-G (Fig 5). It is apparent that the M-G-G substitution cluster in group A and F-D-N substitution cluster in group B are completely

separated from each other, which are strongly associated with *H. pylori* isolates of Asian and non-Asian origins respectively. Based on the process of nucleotide transitions as illustrated in Fig 5 and the contribution of Asian M-G-G substitution type (83%, 143/172) in all isolates studied, it is postulated that *H. pylori* with M-G-G substitution has its origin in Asia. The origin of M-G-G from Asian is explicit in its prevalence (Asian: 143/158, 91%; non-Asian: 29/69, 41%) and the simple process of nucleotide transitions. Furthermore, *H. pylori* infection and the most problematic cases of gastroduodenal disease (i.e. gastric cancer) are more prevalent in Asia. Hence, it further supports the speculation that M-G-G could have originated from Asia.

Besides the affiliation of the two major substitution clusters in their discriminatory capability based on geographical origins (with prevalence of M-G-G cluster in Asian and F-D-N cluster in non-Asian areas) (Table 1 & 4), these two substitution clusters also showed significant association with clinical outcome which is supported by the calculated odds ratio (OR = 4.27) (Table 2). Of these, M-G-G cluster is shown to be associated with PUD as compared to the association of F-D-N cluster to NUD. The link of M-G-G among Asians with PUD and F-D-N in non-Asians with NUD may further highlight that *H. pylori* together with environmental factors are collaboratively contributing to the gastroduodenal disease outcome. The results thus show the association of M-G-G cluster in the Asian group where the PUD is more prevalent.[12] [33] However, this does not exclude PUD outside this geographical regions, rather, it emphasizes the usefulness of the substitution clusters (14^{th} – 16^{th}) of HSP20 as a useful indicator to evaluate the risk of developing into certain gastroduodenal diseases (PUD or NUD).

The conserved *hsp20* DNA sequences in all *H. pylori* strains studied have presented a foundation for its use as epidemiological marker. With these DNA sequences, two phylogenetic groups based on Asian and non-Asian origins were effectively differentiated. This differentiating capability is also displayed by the unique 3-amino-acid substitutions at 14^{th} – 16^{th} residues of HSP20. Furthermore, the 3-amino-acid substitution clusters (M-G-G and F-D-N clusters) show significant discriminating efficiency between PUD and NUD (OR = 4.27). We therefore propose *hsp20* and the novel insertion of 3-amino-acid substitution clusters as a potential epidemiological and gastroduodenal disease differentiating marker of *H. pylori*.

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Competing interest

There are no competing interests for this manuscript.

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Figure legend

Fig 1. The phylogenetic analysis of the 227 *H. pylori* isolates based on *hsp20* (HP0515) DNA sequences. PHYLIP (version 3.6) and ML algorithm were used to conduct the analysis.

A: Lithuanian isolates; Aus: Australian isolates; B: Spanish isolates; Cau: other Singapore isolates; CR: Costa Rica isolates; HK: Hong Kong isolates; I: Indian isolates; J: Japanese isolates; Sin: Singapore isolates; SJM: Peruvian isolates; Swe: Sweden isolates; the groups are indicated as A (Asian) & B (non-Asian). The bootstrap replicates are shown at the nodes, the scale bar represents the substitution rate per site.

Fig 2. The 3-D structure of HSP20 (HP0515) protein predicted by homology modeling.

The position of 14^{th} –16^{th} amino acids was colored red, yellow and green, respectively.

Fig 3. The predicted secondary structure of HSP20 (HP0515) protein.

The position of 3-amino-acid substitution linkages (14^{th} – 16^{th} amino acids) is framed in the box.

Fig 4. The alignment of amino acid sequences of HSP20 with its homologues of other bacterial species. The 3-amino-acid substitution linkage (14^{th} – 16^{th} amino acids) is framed in the box.

Fig 5. The probable process of nucleotide substitution sequence in 14^{th} – 16^{th} amino acids.

Letters in parentheses denote amino acids. Substituted nucleotides are bolded and underlined. Arrows indicate probable process of nucleotide substitutions. The probable substitutions are based on the sequences generated in this study, e.g. at 15^{th} amino acid, for glycine (G), only GGG/GGC were recorded while for 16^{th} amino acid, all these GGT,

GGC and GGG were observed. The figure is merely a proposed process of probable nucleotide substitution.

Table 1. Summary of substitutions at 14th –16th amino acids residues of HSP20

Origin		Substitutions							Total No
		M-G-G	**M-E-G**	**I-G-G**	**M-R-G**	**F-D-N**	**L-N-H**	**F-N-H**	
Asian Countries	**Singapore*** (103)								
	Chinese	66	1	3					
	Malay	8				4			
	Indian	18							
	Others**	2					1		
	Hong Kong (6)	6							
	Japan (43)	37	2	4					
	India (6)	6							
	Sub-total No	143	3	7		4	1		158
Non-Asian Countries	**Peru** (12)	5				6	1		
	Costa Rica (9)	3				5	1		
	Sweden (16)	7	4		1	2		2	
	Spain (14)	6				7	1		
	Lithuania (12)	7	1			4			
	Australia (4)	1	1			2			
	US (26695)						1		
	British (J99)					1			
	Sub-total No	29	6		1	27	4	2	69
	Total No	172	9	7	1	31	5	2	227

* Singapore is a multiethnic nation comprising 3 main racial groups (Chinese, Malays and Indians) and a smaller population of Eurasians. ** These 3 Caucasians are visitors from Western countries living in Singapore (labeled as Cau393, Cau526 and Cau1026 as shown in phylogram, Fig 1).

Table 2. Summary of substitutions and the disease status of *H. pylori* isolates

	Type of substitutions	Diseases status		Odds Ratio
		PUD	**NUD**	**(OR)**
Number of isolate & (Percentage)	**M-G-G**	103 (66%)	53 (34%)	
	M-G-G cluster	112 (65%)	60 (35%)	For PUD: 4.27
	F-D-N	6 (33%)	12 (67%)	
	F-D-N cluster	7 (30%)	16 (70%)	For NUD: 4.27

The data analyzed were based on the disease outcome of 195 *H. pylori* isolates.

PUD: gastric ulcer & duodenal ulcer; NUD: gastritis & non-ulcer dyspepsia.

M-G-G cluster (M-G-G, M-E-G, M-R-G & I-G-G) is positively associated with PUD.

F-D-N cluster (F-D-N, L-N-H & F-N-H) is positively associated with NUD.

Table 3. Comparison of DNA polymorphism between geographical groups

Comparisons	*D* (%)	*Ks*	*Ka*	Ratio of *Ks/Ka*	Reference
A_1 vs. A_1	2.43 ± 0.06	0.094	0.006	15.6	Current study
A_2 vs. A_2	3.38 ± 0.09	0.148	0.005	29.6	Current study
B vs. B	3.69 ± 0.19	0.152	0.009	16.9	Current study
A_1 vs. A_2	4.34 ± 0.11	0.185	0.009	20.5	Current study
A_1 vs. B	6.03 ± 0.17	0.212	0.024	8.3	Current study
A_2 vs. B	5.29 ± 0.17	0.190	0.019	10	Current study
vacA m1 vs. m2	24.9%	0.46	0.246	1.9	Atherton *et al*. (26)
atpD	-	-	-	82.5	Maggi *et al*. (27)
scoB	-	-	-	37.7	Maggi *et al*. (27)
glnA	-	-	-	22	Maggi *et al*. (27)
recA	-	-	-	20.3	Maggi *et al*. (27)

D: percentage of the average number of nucleotide substitutions per site; *Ks*: the mean differences between pairs of strains at synonymous nucleotide position; *Ka*: the mean differences between pairs of strains at non-synonymous nucleotide position.

Table 4. The distribution of various substitutions in geographical groupings

	A_1 (n=119)	**A_2** (n=70)	**B** (n=35)
	Asian (115)	Asian (34)	Asian (4)
	Non-Asian (4)	Non-Asian (36)	Non-Asian (31)
M-G-G	M-G-G: 109	M-G-G: 61	
Cluster	M-E-G: 3	M-E-G: 6	-
(n=187)	I-G-G: 7	M-R-G: 1	
F-D-N		F-D-N: 2	F-D-N: 28
Cluster	-		L-N-H: 5
(n=37)			F-N-H: 2

Fig 1

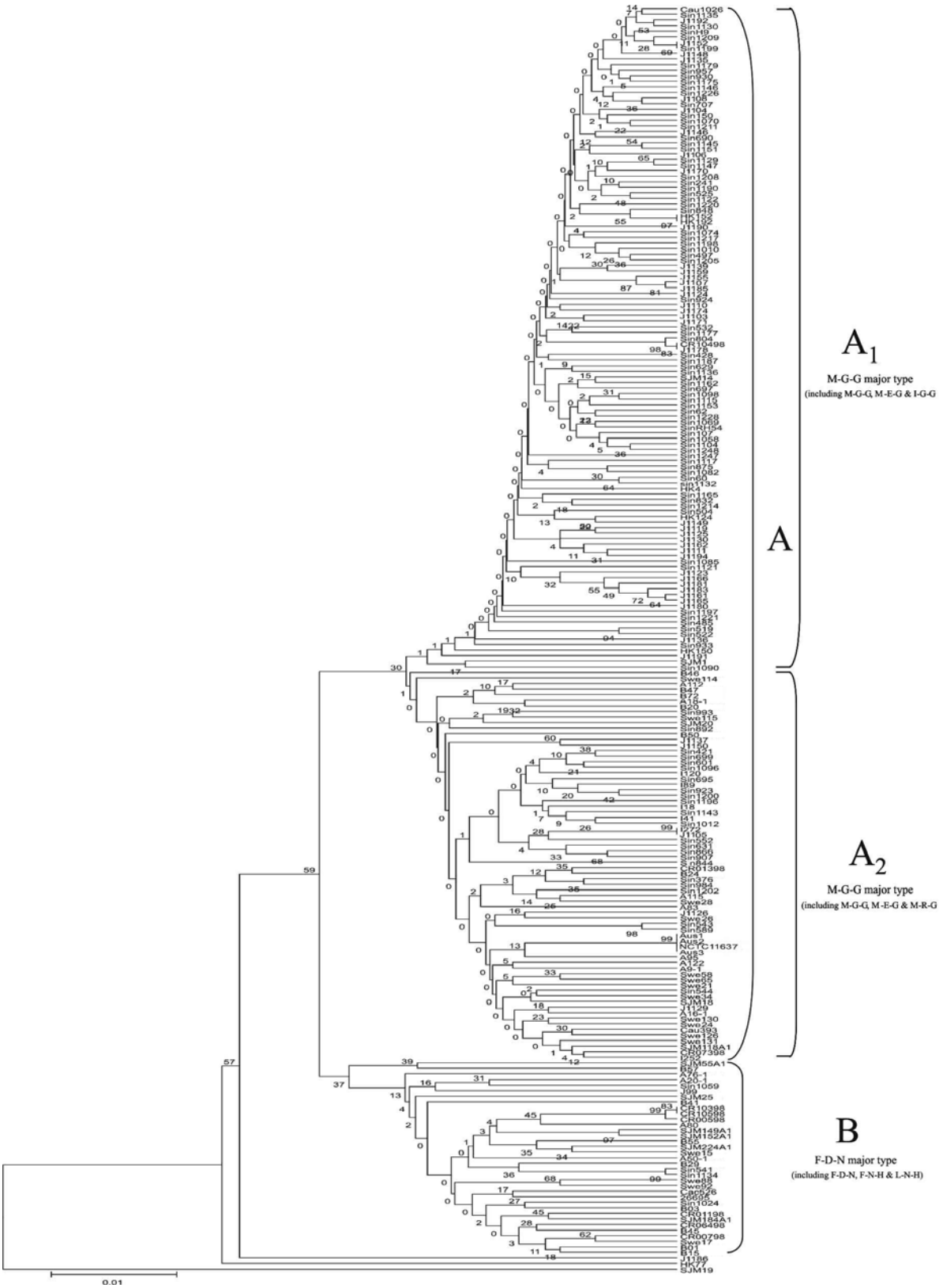

A
A1
M-G-G major type
(including M-G-G, M-E-G & I-G-G
A2
M-G-G major type
(including M-G-G, M-E-G & M-R-G
B
F-D-N major type
(including F-D-N, F-N-H & L-N-H)
0.01

Fig 2

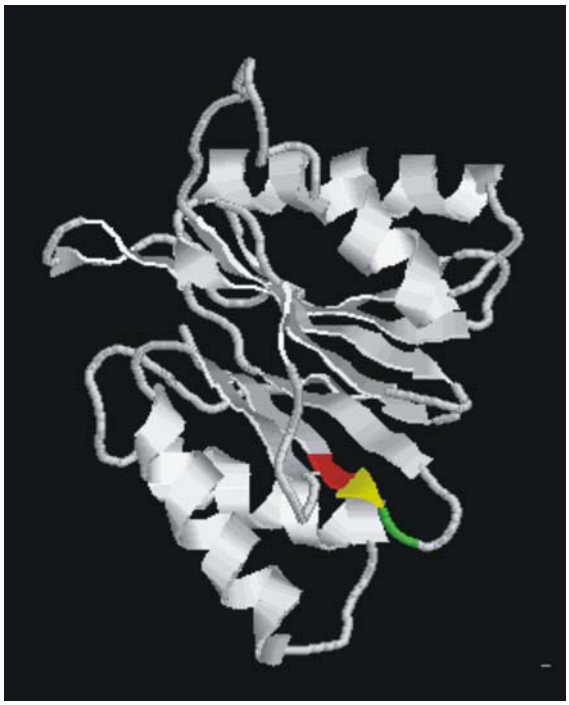
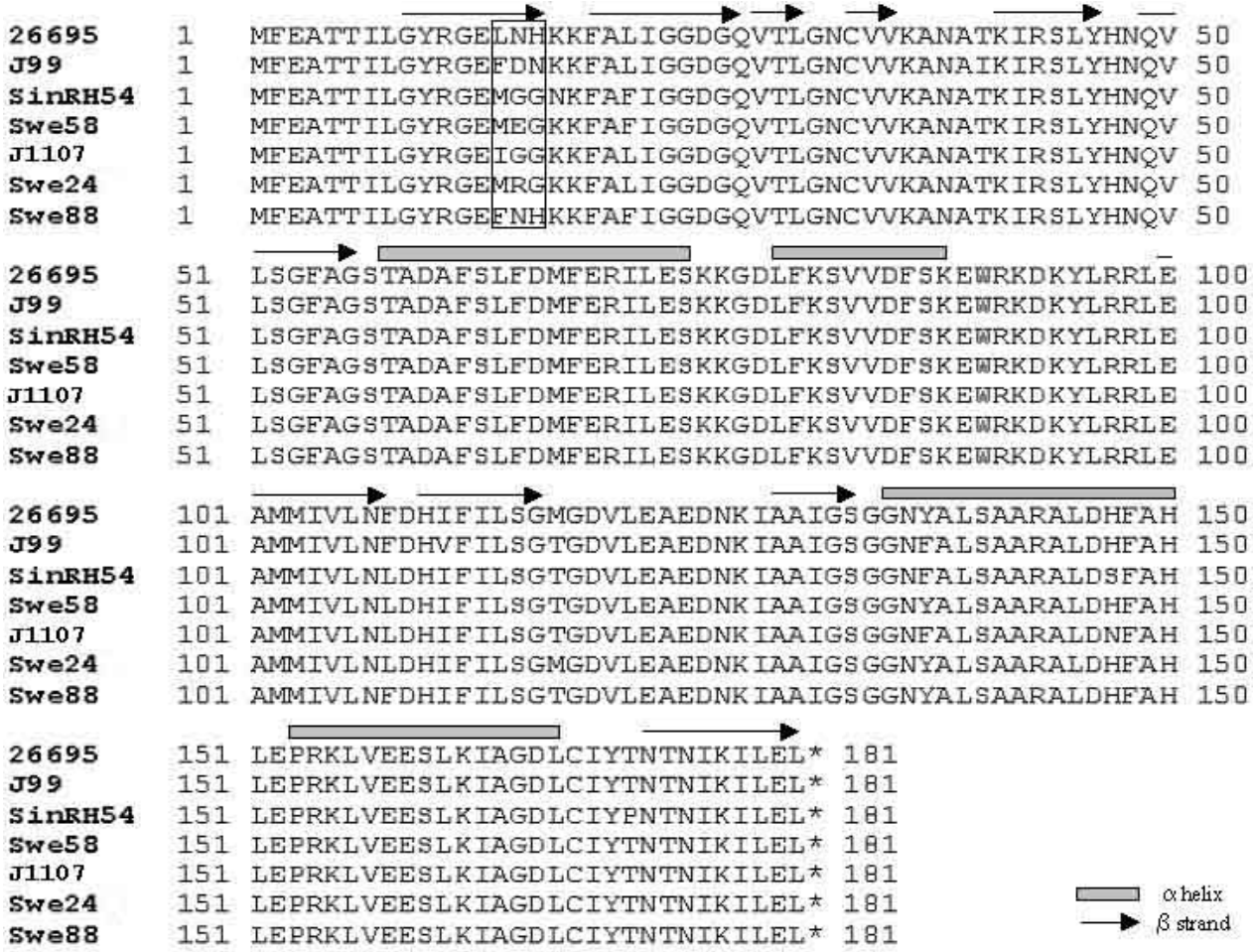


Fig 3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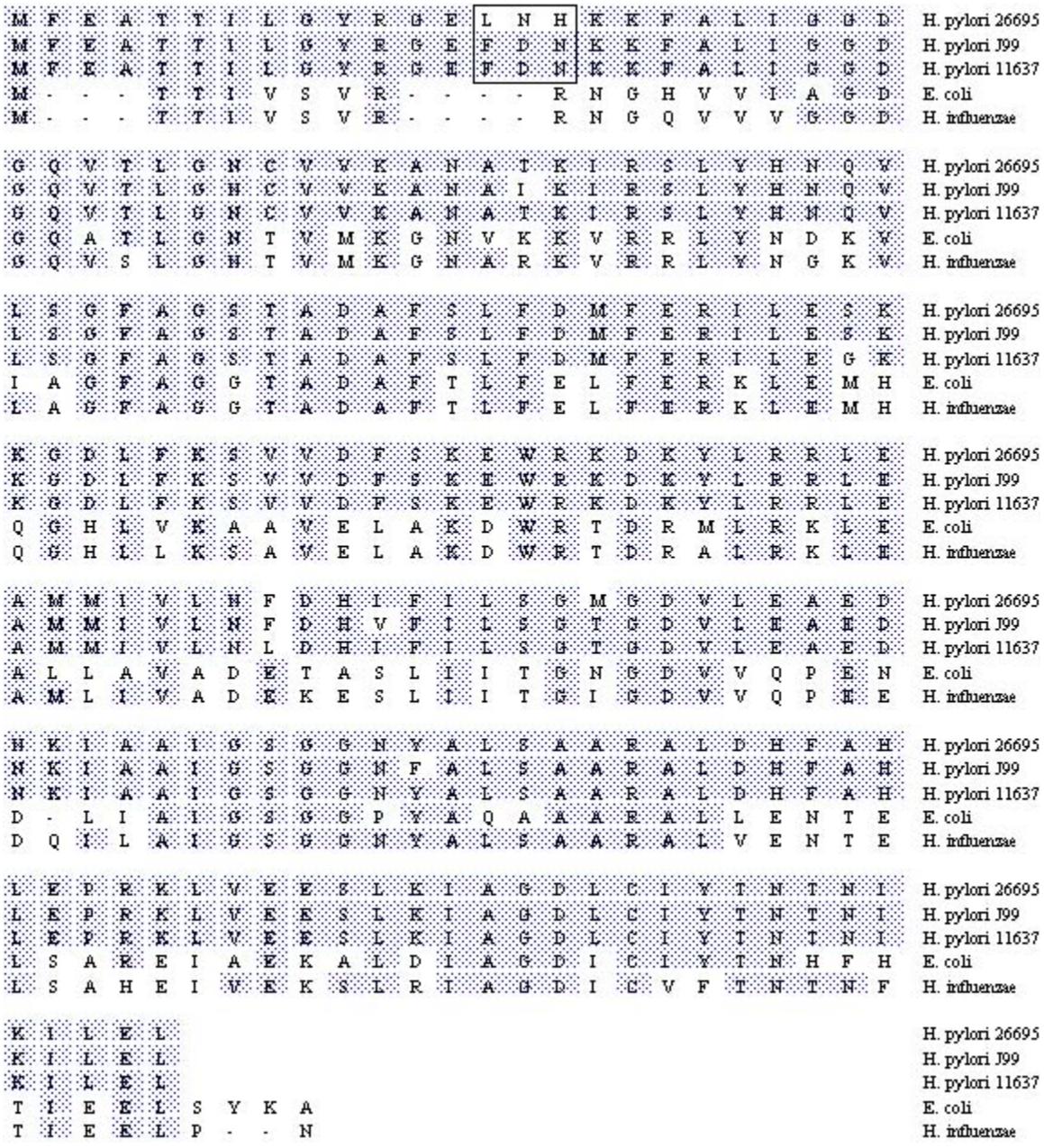
α helix
β strand

Fig 4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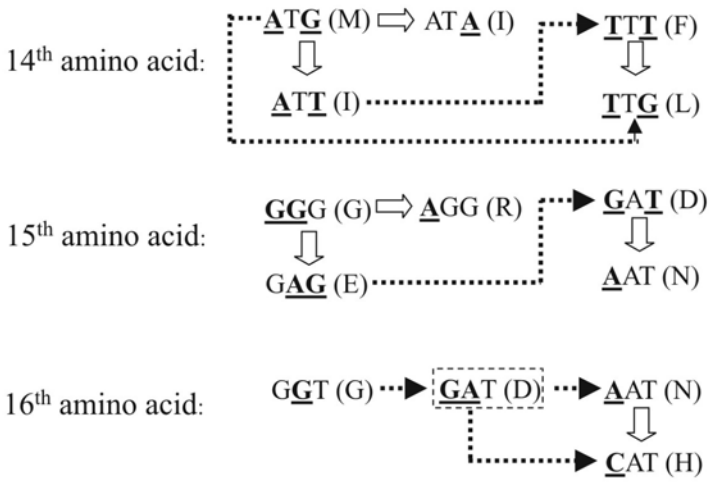
T I E E L P - - N H. influenzae

Fig 5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