RESEARCH ARTICLE

Antigenic Proteins of *Helicobacter pylori* of Potential Diagnostic Value

Akbar Khalilpour[1], Amutha Santhanam[1], Lee Chun Wei[1], Geita Saadatnia[1,2], Nagarajan Velusamy[3], Sabariah Osman[1], Ahmad Munir Mohamad[1], Rahmah Noordin[1]*

Abstract

Helicobacter pylori **antigen was prepared from an isolate from a patient with a duodenal ulcer. Serum samples were obtained from culture-positive *H. pylori* infected patients with duodenal ulcers, gastric ulcers and gastritis (n=30). As controls, three kinds of sera without detectable *H. pylori* IgG antibodies were used: 30 from healthy individuals without history of gastric disorders, 30 from patients who were seen in the endoscopy clinic but were *H. pylori* culture negative and 30 from people with other diseases. OFF-GEL electrophoresis, SDS-PAGE and Western blots of individual serum samples were used to identify protein bands with good sensitivity and specificity when probed with the above sera and HRP-conjugated anti-human IgG. Four *H. pylori* protein bands showed good (≥ 70%) sensitivity and high specificity (98-100%) towards anti-*Helicobacter* IgG antibody in culture-positive patients sera and control sera, respectively. The identities of the antigenic proteins were elucidated by mass spectrometry. The relative molecular weights and the identities of the proteins, based on MALDI TOF/TOF, were as follows: CagI (25 kDa), urease G accessory protein (25 kDa), UreB (63 kDa) and proline/pyrroline-5-carboxylate dehydrogenase (118 KDa). These identified proteins, singly and/or in combinations, may be useful for diagnosis of *H. pylori* infection in patients.**

Keywords: *Helicobacter pylori* - 2-DE - western blot - mass-spectrometry - potential infection markers

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Introduction

Helicobacter pylori is a gram negative, spiral-shaped, microaerophilic, flagellated slow-growing bacteria (Alam et al., 2012). It colonizes the apical side of human gastric epithelial cells and mucous layer (Benoit et al., 2004; Chambers et al., 2013). It was first isolated in 1982 by Marshall and Warren and was recently found to be probably the most chronic bacterial infection in humans (Rieder et al., 2005), infecting more than half of the human population, especially in developing countries (Malaty, 2007). *H. pylori* infections are associated with various gastric diseases. The most common *H. pylori*-associated disease is active gastritis, but the bacteria is also implicated in more severe gastric diseases including chronic atrophic gastritis (a precursor of gastric carcinomas), peptic ulceration, duodenal ulcer and mucosa-associated lymphoid tissue (MALT) lymphomas (Jungblut et al., 2000). The severity of every infection depends on strain virulence, host susceptibility and environmental factors. Some infected patients are symptomatic, whereas some remain asymptomatic for life (Malaty, 2007). However, 6-20% of infected individuals tend to develop duodenal ulceration, and a small proportion of them will develop gastric cancer. *H. pylori* is classified as a class I carcinogen by the World Health Organization (Giannakis et al., 2008). Nevertheless, duodenal ulcers and gastric cancers vary greatly in their symptoms and pathology, and hence, the differences in pathogenesis is a major factor that greatly governs the different patterns of gastritis (Lochhead and El-Omar, 2007). *H. pylori* is also associated with other clinical problems such as asthma and atherosclerotic heart disease (Franceschi and Gasbarrini, 2007). Thus, the early detection and treatment of *H. pylori* infection can reduce morbidity and mortality rates of patients (Chisholm and Owen, 2008).

During *H. pylori* infection, the antigens secreted from the bacterium elicit a strong humoral immune response. Thus, these antigens are regarded as prospective candidates that may serve as infection markers (Robinson et al., 2007). Various *H. pylori* antigens, such as CagA, VacA, HspB, FlaA, FlaB and urease subunits (UreA, UreB), have been employed as diagnostic markers of infection (Widmer et al., 1999; Cremonini et al., 2004;

*[1]Institute for Research in Molecular Medicine, Universiti Sains Malaysia, Minden, [3]Hospital Seberang Jaya, Penang, Malaysia, [2]Department of Biotechnology, Iranian Research Organization for Science and Technology, Tehran, Iran *For correspondence: rahmah8485@gmail.com*

Schumann et al., 2006; Zhang et al., 2012; Flores-Luna et al., 2013; Noto et al., 2013). Although several studies tried to establish an association between well-known virulence markers and clinical outcomes, the results were not conclusive. Some of the markers were used to identify *H. pylori* infection in stool samples (Manes G, 2001; Manes et al., 2001). In some commercially available diagnostic kits, a mixture of antigen extract is used because it provides a higher sensitivity and specificity than assays utilizing a single antigen (Glassman et al. 1990; Andersen and Espersen 1992; Simor et al. 1996; Manes et al., 2005; Pelerito et al. 2006).

In clinical diagnosis of *H. pylori* infection, endoscopy coupled with a urease test is routinely used to detect and evaluate the severity of an infection. Biopsy specimens for culture and histopathology analysis can also be obtained via this method. However, endoscopy is considered costly, for both the hospital and the patient. Endoscopy is also time consuming, requiring set up, preparation of the patient and clean up, in addition to the procedure itself. This is especially problematic for government hospitals in developing countries which have a long queue of patients. Furthermore, the insertion of the endoscope into the stomach often causes patients moderate discomfort.

Detection of *H. pylori* infection markers via serological methods is the easiest non-invasive approach to diagnose the infection, with some tests requiring only a few drops of blood, producing results in less than 5 min. The use of serological tests can reduce the number of patients requiring endoscopy. There are numerous methods available for detection of anti-*H. pylori* IgG, IgA and IgM antibodies, which are present in whole blood, serum, saliva, stool and urine (Faulde et al., 1991; Simor et al., 1996; Miwa et al., 2001; Manes et al., 2005; Sasidharan and Uyub, 2009). The accuracy of the diagnostic markers varies from test to test and among sample types (Vaira and Vakil, 2001). Many ELISA kits for detection of *H. pylori* antibodies have been reported to provide reliable results when used in Western countries (Evans et al. 1989; Crabtree et al. 1991; Jensen et al., 1993; Marchildon et al. 1996; Meijer et al. 1997; Leung et al. 1999). However, when these kits were tested in Asian countries, the diagnostic accuracy was significantly lower (Leung et al., 1999; Miwa et al., 2000; Obata et al., 2003). These differences may be due to several reasons, such as strain heterogeneity in different geographic regions (Ohtsuka et al., 1997; Miwa et al., 2002); cross-reactivity with other intestinal pathogens, which vary in different parts of the world (Graham et al., 1996); and varying immunological responses to *H. pylori* antigens in different patient populations (Khanna et al., 1998).

Thus, it is important to identify *H. pylori* antigens from a local isolate that can be used to detect *H. pylori* infections in the local population with high sensitivity and specificity. These antigens may also be suitable for use in populations in neighbouring regions.

Materials and Methods

Ethics statements

Patient serum samples used were the remaining specimens that had been processed for routine investigations by the diagnostic laboratory at Hospital Seberang Jaya, Penang, Malaysia. Tissue biopsy sampling from patients is a procedure routinely performed by the Endoscopy Unit of the hospital for the urease test. However, for the purpose of this study, an extra piece of tissue was obtained from the patient. Written informed consent was obtained from each patient prior to collection of serum and tissue samples. Approval for the collection of the serum and biopsy samples was obtained from the 'Universiti Sains Malaysia Human Research Ethics Committee' (Ref no: USMKK/PPP/JEPeM [214.3.4]).

Bacterial strain and growth conditions

Cultures of the biopsy tissue samples used to isolate *Helicobacter pylori* were performed on Tryptic Soy Blood Agar with 5% defribinated sheep blood under microaerophilic conditions (10% CO2, 5% oxygen, 85% in air) at 37ºC for 5 to 7 days (Andersen and Espersen, 1992; Chan et al., 2006). Then, the samples were subcultured to isolate single colonies. The isolates were identified by colony morphology, negative Gram staining and positive reactions in biochemical tests for urease, catalase and oxidase. In addition, PCR using published primers was performed to confirm the presence of the CagA and VacA genes.

An *H. pylori* isolate from a patient with duodenal ulcer was used to prepare antigens for 2-DE analysis. The selection was based on the fact that the isolate demonstrated consistently good growth in vitro. This is important because mass-culture of the bacteria was needed for this study.

Serum samples

Venous blood samples of Malaysian patients from the three major ethnic groups were obtained by trained nurses. In the process of identification of potential infection markers, it was important to only use serum samples from patients with confirmed *H. pylori* infections. Thus, samples from patients with positive culture results were considered as confirmed positive sera. In addition, the presence of anti-*H. pylori* IgG antibodies in the serum samples was additional supportive evidence of infection. However, individuals with non-detectable anti-*H. pylori* IgG antibodies were considered as not infected. The serological screening was performed using a commercial *H. pylori* IgG-ELISA kit (Adaltis, Italy).

Serum samples for this study were divided into four categories. Group I patients had tissue biopsy samples positive for *H. pylori* and serum samples positive for anti-*H. pylori* IgG antibodies. The group comprised two patients with duodenal ulcer, five patients with gastric ulcer, 20 patients with gastritis and three patients with normal scope findings (n=30). Group II patients were healthy individuals without any history of gastric disorders (n=30). Group III patients had gastrointestinal complaints and were referred to the endoscopy unit, but were negative for *H. pylori* (n=30). Group IV patients had other diseases such as typhoid, leptospirosis, Escherichia coli septicaemia, shigellosis, Staphylococcus aureus septicaemia and amoebic liver abscess (n=30). Sera

from patients with other diseases were obtained from Hospital Universiti Sains Malaysia (HUSM), Kelantan. The collected sera samples were aliquoted and stored at -20°C. Table 1 shows the characteristics of the age and gender of the individuals whose sera were used in this study.

Preparation of antigen

The bacterial cells were aseptically harvested by adding 3 ml of phosphate buffered saline to each plate and placing the bacterial suspension in a 10 ml centrifuge tube. After centrifugation at 3,000 X g at 4°C for 15 min, samples were washed with 1 ml of phosphate buffered saline containing 40 μl of protease inhibitor (Roche Applied Science). The resulting pellet was resuspended in 40 mM Tris (pH 7.8) containing protease inhibitor at a ratio of 1:2 and vortexed vigorously for 5 min. Lysis of the bacterial suspension was achieved by performing three freeze-thaw cycles using liquid nitrogen and a 37°C water bath. After centrifugation for 15 min at 10,000 X g, the bacterial pellet was resuspended in a sample buffer containing urea, thiourea, DTT and glycerol. Finally, the sample was vortexed well and centrifuged for 15 min at 10,000 X g. The protein content of the supernatant was determined using the RCDC method (BioRad, USA).

Two-dimensional gel electrophoresis

For the first dimension of the 2D gel electrophoresis, the OFF-GEL apparatus (Agilent Technologies) and a 12-well IPG gel strip of pH 3–10 were used according to the manufacturer's instructions. The IPG gel strip was rehydrated in the assembled device with 40 µl of rehydration buffer per well for 15 min prior to sample loading.

A total of 2 mg of the *H. pylori* bacteria preparation was mixed with OFFGEL sample buffer to a final volume of 2 ml, and 150 µl was loaded into each well. The sample was focused with a maximum current of 50 µA, a maximum power of 200 mW and typical voltages ranging from 400 to 4000 V until 64 kVh was reached, after approximately 17 hrs (Baik et al., 2004; Ge et al., 2007). For the second dimension, samples from each of the 12 OFF-GEL fractions were separately mixed with sample buffer (4:1 ratio) containing 28.6% SDS and 4.76% 2-mercaptoethanol. A sample volume of 20 ug per well, for each fraction-sample buffer mixture, was evenly loaded and electrophoresed in a 12% SDS-PAGE gel in running buffer containing 0.3% Tris, 1.44% glycine and 0.1% SDS with a pH of 8.3. Electrophoresis was performed with a constant current of 100 V for 100 min.

Western blotting

Protein bands were electrophoretically transferred from the SDS-PAGE gel onto a 0.45 μm nitrocellulose membrane (NCP) using a semi-dry transblot (Bio Rad USA) at a constant current of 12 A for 30 min. After the transfer, unbound sites on the NCP were blocked for 1 hr at room temperature with blocking solution (Roche Diagnostic) and washed three times in TBS with 0.05% Tween 20 for 10 min. Then, the membrane was cut into strips, and each strip was incubated with individual serum samples at a 1:100 dilution.

Immunodetection was performed with HRP-conjugated monoclonal mouse anti–human IgG antibodies at a dilution of 1:2000 for 1 hr. Substrate development was performed using enhanced chemiluminescence blotting reagent (Roche diagnostics, Germany). The molecular weight of proteins specific to *H. pylori* was determined by referring to commercially available standards.

Initially, all the OFF-GEL fractions were analyzed by Western blotting, with each run using the same serum samples from Group I and Group II. After ten such runs, each with a different Group I and II serum sample, it was evident which fractions contained antigenic bands that were reactive with most of the Group I patients samples and not reactive with Group II samples. Thereafter, only those selected fractions were tested with individual patients and control samples to determine the sensitivity and specificity of each antigenic band. The antigenic bands that reacted with more than 70% of the *H. pylori* patients' sera (Group I) and did not cross-react with more than 90% (Group II, III and IV) of the control groups were selected for further analysis.

MS-compatible silver staining

The silver staining method for mass spectrometry was modified from Shenvchenko et al., (1996). After SDS-PAGE, the gel was incubated in fixing solution for 20 min and immersed in incubation solution for 10 min. Next, the gel was washed with distilled water for 10 min. After a thorough washing, the gel was incubated in sensitizing solution for 1 min, then quickly washed (2 x 1 min) with a large volume of distilled water. Subsequently, silver staining solution was added, and the gel was incubated at 4°C for 20 min. Afterwards, the gel was washed (2 x 1 min) with distilled water. Subsequently, developing solution was added, and the gel was placed on a shaker at low speed. When the solution began to darken, it was replaced with fresh developing solution. Once development was near completion, the solution was replaced with stop solution.

In-gel digestion of proteins and sample clean up

Selected proteins bands were manually excised from the silver-stained gels and transferred into microfuge tubes. An in-gel digestion of the gel piece containing the protein band was performed using the method of O'Connell and Stalts (1997). First, the silver-stained excised gel piece was destained by adding 100 mM sodium thiosulphate and 30 mM potassium ferricyanide in a ratio of 1:1 for 20 min (Baik et al., 2004). Then, the supernatant was discarded and replaced by 100 µl of 200 mM ammonium bicarbonate for 20 min at room temperature. After discarding the ammonium bicarbonate, the gel piece was incubated for 15 min in 50 µl of acetonitrile. The supernatant was then discarded, and the gel piece was rehydrated in 25 µl of 25 mM ammonium bicarbonate for 10 min. After discarding the supernatant, the gel piece was air dried for 1 hour. The protein in the gel piece was enzymatically digested by adding 10 μl of trypsin (10 ng/µl) and incubation on ice for 5 min. Then, the gel piece was immersed in 25 µl of 25 mM ammonium bicarbonate and incubated overnight

at 37°C. Next, 50 µl of acetonitrile was added, and the mixture was left for 20 min at room temperature. The supernatant, which contained the digested protein, was transferred to a fresh microfuge tube and dried using a SpeedVac concentrator. The resulting samples were cleaned up using ZipTipC18 pipette tips (Milipore). Finally, samples from the Zip-Tip were eluted into clean micro-centrifuge tubes and concentrated using a vacuum concentrator until they were completely dry (Eppendorf, USA), at this stage, the samples were ready for MALDI-TOF/ TOF mass analysis (Baik et al., 2004; Chan et al., 2006).

Mass spectrometry analysis and protein Identification

The samples were analyzed using an ABI 4800 MALDI TOF-TOF mass spectrometer at the Protein and Proteomics Centre, Faculty of Biological Sciences, National University of Singapore. GPS Explorer™ software Version 3.6 (Applied Biosystems) was used to create and search files with the MASCOT search engine for peptide and protein identification. Databases from NCBI were used for further analysis of the results.

Results

Figure 1 shows an IgG blot profile that is representative of all fractions (except for fraction 1, which is usually not well-separated and often excluded) using serum samples from a patient infected with *H. pylori* (Group I) and a healthy person (Group II). The antigenic bands were consistently seen in blots of fractions 3 (pI 4.16-4.70), 5 (pI 5.33-5.90) and 7 (pI 6.49-7.00). Both fractions 3 and 5 showed antigenic bands of approximately 25 kDa, and fraction 7 showed antigenic bands of 63 and 118 kDa. Thereafter, the complete set of individual serum samples were analyzed by performing SDS-PAGE and Western blotting using these three fractions. The sensitivity of each antigenic band was determined based on the detection of anti-*H. pylori* antibodies when tested with individual patients' serum samples, and the specificity of each band was determined based on the reactivity with the individual control serum samples from Groups II, III and IV (Table 2). The 118 kDa band had the highest sensitivity, with 100% (30/30) detection of the samples. The 63 kDa band was detected in 83.3% (25/30) of the samples. The 25kDa band from fraction 5 was detected in 73.3% (22/30), while that from fraction 3 was detected in 76.6% (23/30) of the samples. Specificities of all antigenic bands were high (98-100%). Table 3 shows the antigenic bands recognized by each of the four kinds of Group I patients' sera based on the different clinical manifestations. Three of the four kinds of sera had an unequal and small number of samples, thus were unsuitable for statistical comparison. Nevertheless, it can be noted that antibodies to gastritis patients seemed to be more reactive with the 118 kDa band than with the other three bands.

Table 4 shows the identities of the proteins as determined by MALDI-TOF/TOF. Identifications were based on consistent results of MS-MS analysis of at least three samples from different SDS-PAGE gels. In the MS analysis, each proteins score far exceeded the significant cut-off values. Meanwhile, the peptide summary from the MS-MS analysis of each protein showed that at least two unique peptides were identified, and at least two peptides

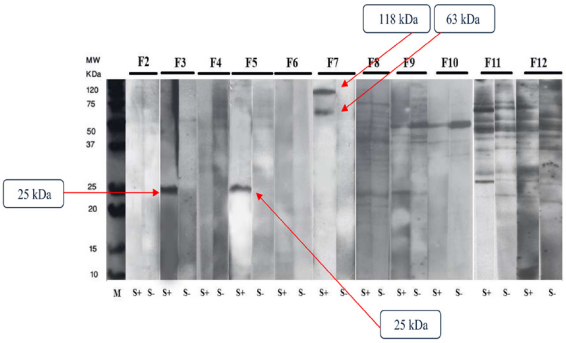

Figure 1. Representative Profile of an IgG Immunoblot of *H. pylori* Antigen Incubated with Patient and Control Serum Samples. M: molecular weight maker; S+: serum from *H. pylori* culture-positive patient (Group I); S-: serum from healthy person (Group II)

Table 1. Characteristics of Individuals Whose Sera Were Used for This Study

Group	No of Patients	Age Range	Average age	No of Males	No of Females
[a]Group I sera	30	18-80	50	17	13
[b]Group II sera	20	22-41	31	6	14
[c]Group III sera	30	17-68	47	15	15
[d]Group IV sera	10	NA	NA	NA	NA

[a]Group I: Patients with biopsy samples that were *H. pylori* culture-positive and serum samples that were positive for anti-*H. pylori* IgG (2 patients with duodenal ulcer, 5 with gastric ulcer, 20 with gastritis and 3 with normal scope findings). [b]Group II: Healthy individuals who had no history of gastric disorders and were seronegative for anti-*H. pylori* IgG. [c]Group III: Patients with biopsy samples that were *H. pylori* culture-negative and serum samples that were negative for anti-*H. pylori* IgG. [d]Group IV: Patients with other infections who were seronegative for anti-*H. pylori* IgG, NA : data not available

Table 2. Results of the IgG Blots of *H. pylori* Antigen Probed with Human Ser

Peroxidise-conjugated secondary antibody	OFF-GEL Fraction No.	MW of Band	Group I[a] (Sensitivity)*	Groups II[b], III[c]&IV[d] (Specificity)**
IgG	3	25 KDa	23/30 (76.6%)	60/60 (100%)
IgG	5	25 KDa	22/30 (73.3%)	60/60 (100%)
IgG	7	63 KDa	25/30 (83.3%)	60/60 (100%)
IgG	7	118 KDa	30/30 (100%)	59/60(98.3%)

*Sensitivity: Number of sera reactive with the band out of the total number of sera tested. **Specificity: Number of sera not reactive with the band out of the total number of sera tested. [a]Group I: Patients with biopsy samples that were *H. pylori* culture-positive and serum samples that were positive for anti-*H. pylori* IgG (2 patients with duodenal ulcer, 5 with gastric ulcer, 20 with gastritis and 3 with normal scope findings). [b]Group II: Healthy individuals that had no history of gastric disorders and were seronegative for anti-*H. pylori* IgG. [c]Group III: Patients with biopsy samples that were *H. pylori* culture-negative and serum samples that were negative for anti-*H. pylori* IgG. [d]Group IV: Patients with other infections who were seronegative for anti-*H. pylori* IgG. MW: molecular weight

Table 3. Antigenic Bands Recognized by Each of the four Kinds of Group I Patients' Sera

Sera from four kinds of patients	No. of patients	Bands 25KDa (Fraction 3)	25KDa (Fraction 5)	63KDa	118KDa
Duodenal ulcer	2	2	2	2	2
Gastric ulcer	5	5	5	5	5
Gastritis	20	15	14	16	20
Normal scope findings	3	1	1	2	3

Table 4. Mass Spectrometric Identification of Antigenic Proteins of *H. pylori*

Fraction No.	Secondary antibody	Bands	Sequence Result					
			Protein ID	Accession Number	Theoretical Mass (Da)	protein score (cut-off score)	No. Peptides (MS-MS)	Theoretical pI
3[a]	IgG	25 KDa	cag island protein [*Helicobacter pylori* J99]	gil15611542	23228	222(>83)	7	5.11
5[b]	IgG	25 KDa	urease accessory protein (ureG) [*Helicobacter pylori* 26695]	gil15611134	22098	462(>83)	6	5.02
7[c]	IgG	63 KDa	urease UreB [*Helicobacter pylori* 26695]	gil5913962	61803	420(>82)	4	5.64
		118 KDa	proline/pyrroline-5-carboxylate dehydrogenase [*Helicobacter pylori* J99]	gil15611119	135778	179(>54)	6	6.22

[a]pI: 4.16-4.70. [b]pI: 5.33-5.90. [c]pI: 6.49-7.0

had individual ion-scores above the cut-off values that indicated identity or extensive homology.

Discussion

Genetic heterogeneity of *H. pylori* has been reported in different geographic regions and within a population. A study of 78 *H. pylori* isolates from three major ethnic groups in Malaysia using multilocus sequence typing (MLST) of seven housekeeping genes and comparison with global *H. pylori* data showed that the isolates can be differentiated into three populations i.e. hpEastAsia, hpAsia2 and hpEurope. Interestingly, the Malaysian Indian and Malay isolates formed a new subpopulation within hpAsia2 i.e. hspIndia. It can be expected that if more isolates or more geographical regions are sampled, more subpopulations of *H. pylori* and populations can be identified (Tay et al., 2009). As mentioned earlier, the diagnostic accuracy of serology kits for detection of *H. pylori* antibodies may be influenced by strain differences in different regions of the world (Leung et al., 1999; Miwa et al., 2000; Obata et al., 2003). Thus in this study a local isolate of *H. pylori* from a duodenal ulcer patient and serum samples from patients with various clinical presentations and from three major ethnic groups in Malaysia were used to identify antigens of potential diagnostic value.

Most of the previous studies on developing diagnostic tests for detection of *H. pylori* infection involved analysis of urease enzyme, VacA, CagA, HspB, FlaA, FlaB and outer membrane proteins (OMPs) (Michetti P, 1999; Zheng J et al., 2002; Cremonini et al. 2004; Schumann et al. 2006; Zhang et al., 2010; Chambers et al., 2013).Other bacterial components, such as heat shock protein, flagella and *H. pylori* adhesin (HpaA), have also been reported as pathogenic determinants (Park et al., 2006). In addition, a few unidentified antigenic bands, with molecular weights of 18, 34 and 39.5 kDa, have been reported to possess good diagnostic value (Andersen and Espersen 1992; Galmiche et al., 2000; Keenan et al., 2000; Haas et al., 2002). Lin et al (2007) detected seven proteins, with relative molecular masses of 76 KDa (flagellar hook protein), 74 KDa [flagellar hook-associated protein (FliD)], 67 KDa (molecular chaperone DnaK), 61 KDa (urease β subunit), 53 KDa (flagellin A), 53 KDa (flagellin B), and 51 KDa (serine protease (HtrA), in serum samples from patients with duodenal ulcers and gastric cancer. In another study, translation elongation factor EF-G (FusA), catalase (KatA) and the alpha subunit of urease (UreA) were reported to be candidate duodenal ulcer-related antigens (Lin et al., 2007). The *H. pylori* GroES gene was also reported to be a novel gastric cancer-associated virulence factor and may contribute to gastric carcinogenesis (Keenan, 2000).

In a comparative study of duodenal ulcer and gastric cancer, Wu et al (2008) identified 15 gastric cancer-related antigens, including threonine synthase, S-adenosylmethionine synthetase, rod-shape-determining protein, peptide chain releases factor 1, DNA-directed RNA polymerase alpha subunit, co-chaperonine GroES (monomeric and dimeric forms), response regulator OmpR and membrane fusion protein; three proteins, elongation factor EF-G (FusA), catalase (KatA) and urease alpha subunit (UreA), were identified as duodenal ulcer-related antigens (Wu et al., 2008). In another study by Alexander et al., (2004), GroEL, HyuA, AtpA and GroES were reported to be potential gastric carcinoma markers (Krah A 2004). In a Korean population-based case-control study, UreB, GroEL, AhpC/TsaA, TagD, FldA and EF-Tu were identified as major protein spots in the 2-DE images of *H. pylori* 26695 (Park et al., 2006).

Proteomics approaches have been used to map protein patterns of multiple *H. pylori* strains, and a number of studies have reported immunoproteomic approaches to identify antigenic proteins (Mini et al., 2006; Krah et al., 2004). Proteome technology using 2-DE gel by ESI–MS-MS has been used to identify four *H. pylori* immunogenic low-molecular-weight (25-30 KDa) proteins: UreA, cell binding factor 2, hypothetical protein and outer membrane protein. The specificity of these proteins was evaluated using both 1-D and 2-D immunoblotting against a group of sera from patients with different bacterial infections (Utt et al., 2002). Thus far, there are no reports of 2-DE antigenic protein analysis of a Malaysian *H. pylori* isolate.

The present work utilized an OFF-GEL apparatus for the first dimension separation because it allowed liquid fractions that showed antigenic bands with good sensitivity in initial experiments to be selected among the 12 fractions. The selected fractions were subsequently used for Western blot analysis to determine the sensitivity and specificity of each antigenic band by probing individual samples from patients and controls. This greatly economized the use of the limited serum samples when compared to the sera usage using traditional gel-based isoelectric focusing.

The results showed four antigenic proteins that were sensitive and highly specific for the detection of *H. pylori* infection. The ~118 kDa band had the highest sensitivity (100%). When the antigenic bands were analyzed against

patient's clinical presentations, this band was observed more frequently with sera from gastritis patients. However, valid conclusions require greater numbers of patients with each type of clinical presentations.

The UreB protein observed in this study has previously been reported to be antigenic (Park JW 2006; Wu et al., 2008). It demonstrated a strong antigenicity and protection among known H pylori proteins and showed 95% sequence identity among all *H. pylori* isolates (Mao and Yan 2004). Although UreB has been reported to be an antigenic protein (Voland et al., 2002; Ruiguang Ge., 2007; Park et al., 2008), there are no previous reports of its sensitivity rate for detecting *H. pylori*.

To the best of our knowledge, this is the first report to identify CagI, UreG, and proline/pyrroline-5-carboxylate dehydrogenase as *H. pylori* antigenic proteins. CagI has been described as a putative surface protein based on its tendency to interact with integrin (Jiménez-Soto LF, 2009). An N-terminal fragment of CagA, a C-terminal fragment of CagY and intact CagI were shown to bind to β1 integrin. Integrins are highly conserved and are reported to play a key role in cell migration, cell–cell adhesion, cell–extracellular matrix adhesion, proliferation, phagocytosis, apoptosis and differentiation (Kwok et al., 2007). UreG is one member of the Ure gene cluster, and it encodes an accessory protein required for nickel ion insertion into the apoenzyme (Mobley, 1996; Mao and Yan, 2004). In the biosynthesis of the active metal-bound form of the nickel-dependent enzyme urease, a lysine-carbamate functional group is formed alongside with the delivery of two Ni (2+) ions into the precast active site of the apoenzyme. UreG functions as a chaperone in the urease active site assembly and is often required to complete the biosynthesis of nickel-enzymes (Zambelli et al., 2009). Pyrroline-5-carboxylate dehydrogenase plays a key role in the metabolic pathway, as it catalyzes the oxidation of proline to glutamate in the presence of NAD (Inagaki et al., 2006; Parsons et al., 2011).

In most commercial diagnostic kits, a mixture of antigenic extracts is used because it provides higher sensitivity and specificity than a single antigen (Glassman et al., 1990; Andersen and Espersen 1992; Simor et al., 1996; Manes et al., 2005; Pelerito et al., 2006). However, the identities of the antigens used in commercial kits are usually not disclosed. Thus, mixtures of various combinations of antigens identified in this study may be tested for their usefulness in detection of *H. pylori* infection.

Future studies will involve recombinant forms of each protein and much larger sample size. In addition, post-treatment samples will be performed to assess the decline in antibody levels to these proteins because persistence of a low level of antibodies has been reported after eradication of *H. pylori* (Bergey B, 2003).

In conclusion, four *H. pylori* infection markers identified in this study showed good sensitivity and high specificity, and three of them have not previously been reported to be antigenic. Recombinant forms of these proteins, either singly or in combinations, are potentially useful for development of a diagnostic test for *H. pylori* infection, particularly for patients in Malaysia, and possibly the immediate region.

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