HELICOBACTER PYLORI INFECTION IN PAEDIATRIC PATIENTS WITH DYSPEPTIC SYMPTOMS

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NATIONAL UNIVERSITY OF SINGAPORE

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HELICOBACTER PYLORI INFECTION IN PAEDIATRIC PATIENTS WITH DYSPEPTIC SYMPTOMS

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“In a world of black and white, *Helicobacter pylori* is gray”

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- **BL Ng**, SH Quak, M Aw, KT Goh, B Ho. Immune Response to Differentiated forms of *Helicobacter pylori* in Children with Epigastric Pain. Clin Diagn Lab Immunol. 2003 Sep;10(5):866-9.

- **BL Ng**, SH Quak, B Ho. Conservation of vital genes and proteins for the survival of the dormant coccoid form of *Helicobacter pylori*. Submitted.

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- SH Quak, **BL Ng**, HC Ng, B Ho. Seroprevalence of *Helicobacter pylori* infections in Singaporean Children with Dyspepsia and Characterization of the Clinical Isolates. Planetary presentation at the International Symposium on Helicobacter and Viral Hepatitis, Singapore, 15-17 Feb 2001. Proceeding p22.

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PUBLICATIONS

ABBREVIATIONS

AGE	**Acid glycine extract**
AST	**Antimicrobial susceptibility test**
babA2	**blood group antigen-binding adhesin**
BHI	**Brain heart infusion**
BSA	**Bovine serum albumin**
CFA	**Complete freunds adjuvant**
CO_2	**Carbon dioxide**
DIGE	**Difference gel electrophoresis**
DMF	**Dimethylformamide**
EHPSG	**The European *Helicobacter pylori* Study Group**
ELISA	**Enzyme-linked immunosorbent assay**
E-Test	**Epsilometer test**
FISH	**Fluorescent in situ hybridisation**
FITC	**Fluorescein isothiocyanate**
cagA	**cytotoxic associated gene**
GAM	**Goat anti-mouse**
HpSA	***Helicobacter pylori* stool antigen test**
IARC	**International Agency for Research on Cancer**
iceA	**induced by contact with epithelial cells**
IEF	**Isoelectric focusing**
IgG	**Immunoglobulin G**
Le	**Lewis**
LPS	**Lipopolysaccharide**
LVER	**Low Viscosity Epoxy Resin**
Mabs	**Monoclonal antibodies**
MALDI-TOF	**Matrix Assisted Laser Desorption Ionization Time–of-flight**
MIC	**Minimium inhibitory concentration**
MW	**Molecular weight**
NSAIDs	**Non-steroidal anti-inflammatory drugs**

ABBREVIATIONS (Con't)

NUD	**Non-ulcer dyspepsia**
OD	**Optical density**
PBS	**Phosphate-buffered saline**
PBST	**PBS containing 0.05% Tween 80**
PCR	**Polymerase chain reaction**
PVDF	**Immibilon polyvinylidene difluoride R**
RAP	**Recurrent abdominal pain**
RAPD	**Random Amplified Polymorphic DNA**
RFLP	**Restriction fragment length polymorphism**
ROC	**Receiver operating characteristic**
ROS	**Reactive oxygen species**
SDS-PAGE	**Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis**
TEM	**Transmission Electron Microscopy**
UBT	**Urea breath test**
ureA	**urease A**
ureC	**urease C**
vacA	**vacuolating cytotoxin A**
VBNC	**Viable but non-culturable**
1D PAGE	**One dimensional polyacrylamide gel electrophoresis**
2D PAGE	**Two dimensional polyacrylamide gel electrophoresis**

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SUMMARY

Helicobacter pylori has shown strong association with various gastroduodenal diseases. It has been reported that *H. pylori* infection is acquired during childhood and may persist for decades but its mode of transmission has yet to be established.

H. pylori differentiates morphologically from spiral to coccoid form during prolonged incubation, yet exhibited similar DNA fingerprints throughout this conversion. By immunogold labelling transmission electron microscopy, using antibodies raised against the spiral (3 day old culture) and coccoid (150 day old culture) antigens, results showed that these antigens were localized on the surface of *H. pylori*. Flow cytometry revealed that both antigens shared common immunogenic epitopes and possibly respective unique proteins that hitherto not isolated/ identified.

Sera of 21 families comprising 72 members when examined by ELISA and western blotting using the 3 day old spiral antigen showed that seropositivity was more prevalent in the older family members. Comparison of the western blot profiles showed highly similar protein patterns between seropositive family members residing in the same household. This study demonstrates the possibility of an intrafamilial vertical transmission and *H. pylori* infection could possibly begin at young age.

In the follow-up serology study, seroprevalence against *H. pylori* spiral or coccoid antigens in the paediatric children with epigatric pain (n=489) was 2 fold higher than the asymptomatic school children (n=599). Interestingly, there was a 4 fold increase in IgG levels to the coccoid antigen as compared to the spiral antigen among the symptomatic or control group. The results imply that the coccoid form may have a role in *H. pylori* infection in the symptomatic young population. Western blot profiles of representative seropositive and seronegative sera against both forms of antigens further show that the

coccoid form of *H. pylori* is capable of eliciting a humoral immune response that is highly similar to that induced by the spiral form. This finding raises a concerned question: Is there an under-reporting on the presence of *H. pylori* infection in this group of young patients?

The 15 isolates from different paediatric patients with epigastric pain presented different genetic fingerprints by RAPD. The PCR analysis of different virulence genes showed that the *H. pylori* strains in Singapore are different from the western countries. The prevalence of antibiotics resistance and the Lewis antigens expression are similar to the local adult population.

As morphological conversion progressed, there was a reduction of protein content. Comparative differential analysis on 1D and 2D PAGE demonstrated that the coccoid form has retained "essential" proteins which had been identified as Translational elongation factor EF-TU, 26 kDa, non-heme containing ferritin (Pfr) and neutrophil activating protein (Nap*A*) by mass spectrometry (MALDI-TOF). *cagA* and *vacA* were shown to be conserved by PCR. In addition, the expression of *26 kDa*, *ureA* and Lewis antigens were maintained throughout the time course study.

The significant increase in seroprevalence to the coccoid antigen as compared to the spiral antigen in the symptomatic children suggests that the infection possibly could have begun with the coccoid form. Strong evidence on vertical transmission was shown in the intra-familial study. The proteomic and genetic studies indicated that most genes were conserved during the morphological conversion. There are signs of conserved property of the 2 forms. Present comparative epidemiological and proteomics studies have lend

support to the notion that coccoid form is viable and likely to have a role in the transmission of *H. pylori*.

INTRODUCTION

Helicobacter pylori infection is one of the most common chronic infections in human since the isolation of the pathogen by Warren and Marshall (1983) two decades ago. This gram-negative gastric bacterium chronically inhabits the stomach of more than half of the world population (Parsonnet, 1999; Westblom *et al.*, 1999) and is associated with the development of acute chronic gastritis, peptic ulcer disease, gastric adenocarcinoma and mucosal-associated lymphoid tissue (MALT) lymphoma (Correa & Miller, 1995; Kuipers *et al.*, 1995; Mitchell, 1999). Most of the infected individuals remain asymptomatic throughout their life whilst only about 10% of those infected develop peptic ulcer disease and fewer than 1% are likely to develop gastric carcinoma (Jones & Sherman, 2001).

1. 1 *H. pylori* INFECTION BEGINS WITHIN FAMILIES

Despite the extensive studies on *H. pylori* infection, the precise mechanism of transmission has not been established. Infection occurs predominantly through household contact during childhood (Malaty *et al.*, 2000) while studies by Elitsur *et al.* (1999) and Taneika *et al.* (2001) have demonstrated intrafamilial clustering of *H. pylori* infection. The studies on family aggregation of the infection provide a model for investigating transmission and the natural history of initial infection.

1.2 *H. pylori* INFECTION IN PAEDIATRICS

1.2.1 Prevalence during childhood

Reports have shown that childhood is a time of high risk for acquiring *H. pylori* infection (Lindkvist *et al.*, 1996; De Giacomo *et al.*, 2002). Boey and co-workers (1999)

conducted an epidemiological study on the prevalence of *H. pylori* infection in 514 asymptomatic children. Of the 261 children in age group of 0.5-5 years old, 19 (7%) were found to be seropositive for *H. pylori*. A recent report by Malaty *et al. (*2002) which studied the age acquisition of *H. pylori* infection from infancy to adulthood, demonstrated that the highest rates of acquisition of the infection was before the age of 10. Although serious complications associated with the infection are rare in childhood, disease progression may in the long run lead to peptic ulcer disease or gastric carcinoma in a small proportion of those infected (Forman *et al.*, 1991; Imrie *et al.*, 2001).

1.2.2 Association with recurrent abdominal pain (RAP) and epigastric pain

RAP is defined as the occurrence of three or more episodes of abdominal pain of such severity as to interfere with a child's normal activity over a 3 months period (Apley & Naish, 1958). This phenomenon is one of the most common complaints in childhood and the prevalence of organic diseases responsible for the pain has ranged from less than 10 % (Stone & Barbero, 1970) to more than 50 % (Ashorn *et al.*, 1993). Epigastric pain is one of the main presenting symptoms associated with abdominal pain (Uc & Chong, 2002). While *H. pylori* infects the younger population, its association with the development of non-ulcer dyspepsia and pathogenesis of RAP has been less clear (Heldenberg *et al.*, 1995; Walsh *et al.*, 1997; Bode *et al.*, 1998). There are studies which showed an association between RAP and *H. pylori* infection in children (Chong *et al.*, 1995; Rutigliano *et al.*, 1999; Frank *et al.*, 2000) whereas there are others which demonstrated otherwise (Bode *et al.*, 1998; Wewer *et al.*, 1998; Macarthur, 1999).

1.2.3 Treatment and antimicrobial resistant *H. pylori*

The standard regimen most widely used to eradicate *H. pylori* is the triple therapy, which consists of 2 antibiotics and bismuth (Logan *et al.*, 1991; Graham *et al.*, 1992) or a proton pump inhibitor (Bazzoli & Pozzato, 1997). The antibiotics potentially useful in *H. pylori* eradication are clarithromycin, metronidazole, amoxicillin and tetracycline. Guidelines proposed by the Canadian *Helicobacter* Study Group for the management of paediatric *H. pylori* infection recommend diagnostic tests only in children who are most likely to benefit from treatment, such as those likely to have peptic ulcer disease (Sherman *et al.*, 1999). In a separate report from the European Paediatric Task Force, there was consensus that the physician should offer treatment for the infection if the child undergoes endoscopy and *H. pylori* is identified (Drumm *et al*., 2000).

Failure to eradicate *H. pylori* due to antibiotic resistant-*H. pylori* has been a growing clinical problem. The study by Kalach *et al.* (2001), which evaluated the antibiotic resistance in children before treatment of *H. pylori* infection reported the resistance to metronidazole to be as high as 43% and 21% were resistant to clarithromycin. Similarly, the findings from the study of Kato *et al.* (2002), indicated that a high prevalence of clarithromycin resistant strains in Japanese children were associated with eradication failure. In a 9-year study on Spanish children, Lopez-Brea *et al*. (2001) showed that the resistance to clarithromycin and metronidazole in *H. pylori* had increased over the years from 2.27% to 28.33% (for clarithromycin) and 7.14% to 43.9% (for metronidazole).

In Singapore, the prevalence of antibiotic resistance in paediatric patients with *H. pylori per se* has not been evaluated. However, previous work by our research group

has documented an increased prevalence of resistance to metronidazole in *H. pylori* positive patients from 20% in 1995 to 47% in 1998 (Hua *et al.*, 2000a) and hovered at ~50% by 2000 (unpublished data).

1.2.4 Population diversity of *H. pylori*

Genomic diversity of *H. pylori* strains was displayed using methods such as PCR-restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD)-PCR (Hua *et al.*, 1998; Gzyl *et al.*, 1999). The *H. pylori* isolates from unrelated individuals have shown to exhibit different genetic fingerprints.

Several bacterial factors notably *cagA* (cytotoxin-associated gene), *vacA* (vacuolating cytotoxin), *iceA* (induced by contact with epithelial cells) and *babA2* (blood group antigen-binding adhesin) have been implicated in the pathogenicity of *H. pylori* and different genotypes have shown distinct geographical distribution and correlation with disease outcomes (van Doorn, 1998; 1999, Zheng *et al.*, 2000). The *cagA* positive *iceA1 vacA* s1c-m1 genotype was more dominant in Japan and Korea (Yamaoka *et al.*, 1999). However, in the United States, the predominant genotype was *cagA* positive *iceA2 vacA* s1b-m1 genotype. A comparison study by Oleastro and co-workers (2003) on the prevalence of virulence genotypes, namely *cagA*, *vacA* and *babA2* in *H. pylori* isolated from adult and children patients reported that *H. pylori* strains harbouring the more virulent genotypes, particularly the type I (*vacA* s1 and *cagA* positive) and triple positive (*vacA* s1, *cagA* and *babA*2 positive) strains were more prevalent in adults. In another study based on the Mexican population, Gonzalez-Valencia *et al*. (2000) observed differences in strains isolated from adults and children in the same population. Among the

adults in Mexico, they found principally *cagA* positive *vacA* s1b strains as compared to *cagA* negative *vacA* s2 strains in children.

1.3 COCCOID FORM OF *H. pylori*

H. pylori has been found to occur in two morphological forms: spiral and coccoid. The actively replicating spiral form transforms into the dormant coccoid form when exposure to stressful environmental conditions such as increased oxygen tension (Catrenich & Makin, 1991; Cellini *et al.*, 1994a), acid stress (Mizoguchi *et al.*, 1998), treatment with antimicrobial agents (Berry *et al.*, 1995) as well as prolonged incubation (Hua & Ho, 1996). The spiral form has been shown to be the infective form that is responsible for gastroduodenal diseases as there is no definitive role dedicated to the coccoid form to date.

The coccoid form of *H. pylori* is not culturable *in vitro* and the study by Kuster *et al.* (1997) suggested that this form is the morphological manifestation of cellular degeneration and cell death. In the study, the total amounts and integrity of the RNA and DNA were significantly reduced and absence of a membrane potential were observed. Similar conclusion was also drawn from a number of other studies (Narikawa *et al.*, 1997; Monstein *et al.*, 1998), showing non-random fragmentation of ribosomal RNA and degradation of nucleic acid as the culture aged. On the contrary, other investigators had demonstrated that the coccoid form is viable but non-culturable (VBNC) as reports suggested that coccoid form maintained cell structures, metabolism and protein expression (Cellini *et al.*, 1998; Monstein & Jonasson, 2001; Nilsson *et al.*, 2002).

Experimental data had shown the survival of *H. pylori* coccoid form in water for months (West *et al.*, 1990; Mai *et al.*, 1991). Moreover, animals infected with the coccoid form were reported to be able to colonize and produce gastric alterations (Cellini *et al.*, 1994b; Wang *et al.*, 1997). A recent study by Rabelo-Goncalves *et al.* (2002) describing the histopathological findings in BALB/c mice model, challenged with coccoid strain of *H. pylori*, showed induction of an acute inflammatory response in the stomach of reinfected mice. The coccoid form was also present on gastric mucosa of infected individuals (Chan *et al.*, 1994).

The mode of transmission of *H. pylori* infection has yet to be unravelled. However, the coccoid form, which is the potential survival of extragastric form, has been suspected to play a vital role in the transmission process. This form was also implicated in relapses of infection after the antimicrobial treatments (Brenciaglia *et al.*, 2000)

1.4 OBJECTIVES

The objectives of this study are:

Epidemiological studies

1) To study the possible intrafamilial transmission of *H. pylori.*

2) To examine the seroprevalence of *H. pylori* in local paediatric patients with epigastric pain and to compare the level of serum IgG against both the spiral and coccoid forms of *H. pylori*.

3) To characterize the clinical isolates from symptomatic paediatric patients with respect to genetic diversity, antibiotic susceptibility, Lewis antigens expression and presence of the putative virulence determinants.

Dimorphism of *H. pylori*

1) To study the DNA fingerprint, analyse the virulence genes and Lewis antigens expression during morphological conversion from spiral to coccoid form.

2) To compare the protein expression profiles during the morphological conversion from the spiral to coccoid form by proteome tools and to identify the proteins of interest (upregulated/downregulated) using mass spectrometry.

SURVEY OF LITERATURE

2.1 HISTORY

The first well known report of gastric *Helicobacters* has been credited to an Italian anatomist Giulio Bizzozero, as early as in 1893. In hand drawn illustrations, Bizzozero documented the presence of “spirochetes” with approximately 10 wavelengths within the parietal cells and gastric glands in the stomachs of dogs. However, the first record on the presence of spiral organisms in the human mucosa, adjacent to carcinomas was described by Krienitz (1906).

In 1939, Doenges showed 43% of human stomach autopsies harboured spiral organisms and a year later, Freedberg and Baron (1940) presented findings of “spirochetes” in about 40% of the resected gastric specimens. These findings were viewed with scepticism as most of the samples of spiral organisms were obtained *post mortem* and the possibility of contamination could not be disregarded. Moreover, the hypothesis of contamination gained superiority in the early 1950s when Palmer performed a study on more than 1,000 gastric biopsies taken with a blind suction biopsy instrument and found no evidence of spirochetes. This incorrect conclusion drawn during that period could be due to the rigid endoscopes available, which only allowed biopsies to be taken from the fundus and not from the antrum, where *H. pylori* is usually located. The possibility that the appropriate staining solutions were not used could not be ruled out.

The interest in this gastric spiral bacterium was re-awakened when Steer and Colin-Jones (1975) noted that numerous spiral bacteria were present in 80% of their gastric ulcer specimens. Unfortunately, attempts to culture the organism yielded only growth of *Pseudomonas aeruginosa*. This was later assumed to be the contaminants from the endoscope.

A major breakthrough in locking the link between gastroduodenal diseases and the spiral bacteria was established when two Australian researchers Robin Warren and Barry Marshall (1983), after numerous unsuccessful attempts, managed to culture the *Campylobacter*-like organism (CLO) by chance. Furthermore, Barry Marshall himself had fulfilled the Koch's postulate, confirming an association of the bacteria and clinical disease by voluntarily ingesting a culture of *H. pylori* and was subsequently diagnosed with gastritis (Marshall *et al.*, 1985a).

The brief history on the discovery of *H. pylori* as illustrated in Table 2.1 shows that *H. pylori* has existed all this while, but investigators were not able to detect the bacteria in biopsies or merely considered their findings a result of contamination. The discovery of the gastric pathogen, *H. pylori*, has indeed led to a revolution in our understanding of gastroduodenal pathology, mainly gastritis and peptic ulcer.

2.2 TAXONOMY AND MORPHOLOGY

2.2.1 Taxonomy of *H. pylori*

This novel gastric bacterium isolated resembled *Campylobacter* in several aspects, both morphologically and microbiologically. Therefore, the primary isolate was first referred to as *Campylobacter pyloridis* (Marshall & Warren, 1984). The specific epithet was later revised to *C. pylori* conforming to the correct Latin genitive of the noun pylorus (Marshall & Goodwin, 1987). However, further characterization of the organism indicated that perhaps *C. pylori* was not a true *Campylobacter*. The ultrastructure details showed multiple sheathed flagella at one pole of the bacterium, in contrast to the single bipolar unsheathed flagellum typical of *Campylobacter* species (Goodwin *et al.*, 1985).

TABLE 2.1 The story of the discovery of *H. pylori*

The story of ***H. pylori***		
Year	**References**	**Report**
1893	Bizzozero	Spiral organisms in dogs
1896	Salomon	Spiral organisms in dogs and cats
1906	Krienitz	First description in a human with gastric cancer
1938	Doenges	Spirochetes in stomach (autopsies)
1940	Freedburg & Barron	Spirochetes in stomach with ulcers or carcinoma
1954	Palmer	All bacteria in stomachs believed to be contaminants
1975	Steer & Colin-Jones	Bacteria in gastric ulcer patients identified as *Pseudomonas aeruginosa*
1983	Warren & Marshall	First culture of *H. pylori*

The protein patterns and cellular fatty acid composition of *C. pylori* also differed markedly from those of *Campylobacter* species (Pearson *et al.*, 1984; Goodwin *et al.*, 1985). Analysis of the 16S rRNA sequence provided more evidence to exclude *C. pylori* from the *Campylobacter* genus (Romaniuk *et al.*, 1987). Finally, Goodwin *et al.* (1989) proposed the new genus name *Helicobacter* and since then, *C. pylori* was renamed *Helicobacter pylori,* the first member of the new genus.

2.2.2 Morphology and physiology of *H. pylori*

H. pylori is a helical S shaped gram negative bacterium. It is 2.5-5 µm in length, 0.5-1 µm in width and possesses a tuft of 4 to 6 polar sheathed flagella (Goodwin *et al.*, 1985). Each flagellum is 2.5 µm long and about 30 nm in thickness, with a membranous terminal bulb (Goodwin *et al.*, 1989). The characteristic corkscrew motility enables the bacterium to burrow into the mucin lining the epithelial mucosa of the stomach (Goodwin *et al.*, 1985). The flagella components consist of the hook protein (Flg E) and two flagellin proteins, FlaA and FlaB. *H. pylori* with disrupted *flgE* were non-motile and lacked the filaments, although both flagellin proteins were produced (O'Toole *et al.*, 1994). Both flagellin subunits were found to be essential for motility and colonization of the stomach (Josenhans *et al.*, 1995).

H. pylori prefers a microaerophilic environment with 5-10% carbon dioxide environment for *in vitro* culture. A variety of solid media containing 5-10% horse/sheep blood were used to culture the bacteria (Hachem *et al.*, 1995). Under microaerophilic atmosphere, *H. pylori* colonies usually appeared after 3-5 days incubation at 37°C.

H. pylori presents 2 different morphologic manifestations: spiral and coccoid forms. The role of the spiral form has been shown to be strongly associated with gastroduodenal diseases (Cover & Blaser, 1992; Annibale *et al*., 2001). However, the biological significance of the coccoid form, which is non-culturable *in vitro,* has yet to be determined (Hua & Ho, 1996; Zheng *et al.*, 1999). Some investigators postulated that the coccoid form may represent a persistent form in which *H. pylori* can exist in the environment as VBNC (Monstein & Jonasson, 2001; Nilsson *et al.*, 2002) and could possibly play a role in the transmission cycle and treatment failure (Axon & Moayyedi, 1996; Cave, 1997).

2.3 *H. pylori* INFECTIONS AND CLINICAL CONSEQUENCES

H. pylori is the known major human bacterial pathogen responsible for gastroduodenal diseases. Once acquired and colonized, it can persist for life in the stomach. Many of those infected in the population experience no apparent adverse clinical consequences. However, a small population of carriers develop acute chronic gastritis, peptic ulcer disease, gastric adenocarcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma (Correa & Miller, 1995; Kuipers *et al.*, 1995; Mitchell, 1999).

2.3.1 Non-ulcer dyspepsia (NUD)

NUD or functional dyspepsia is defined as persistent upper abdominal pain or discomfort in patients without detectable abnormalities in structural or biological examinations (Fisher & Parkman, 1998; Talley *et al*., 1999). In subjects with dyspepsia, endoscopic studies have reported that 15-20% have peptic ulcer, 5-15% gastro-

oesophageal reflux, <2% gastric cancer while majority (60%) have NUD. It was observed that NUD occurs more frequently in younger patients (38% in patients younger than 25 years old) compared with older patients (3%-7% in patients older than 60 years old) (Lockhart *et al.*, 1985; Fobat *et al.*, 1987).

2.3.2 Peptic ulcer disease

Epidemiological studies of *H. pylori* infection have provided evidence demonstrating the implication of *H. pylori* in the development of peptic ulcer. The prevalence of *H. pylori* infection in duodenal ulcer patients is about 70-98.9% (Marshall *et al*., 1985b; Nomura *et al*., 1994; Hu *et al.*, 1995). The causative relationship between *H. pylori* and peptic ulcer is reinforced when the organism was successfully eradicated in patients suffering from duodenal ulcer and the follow-up study over the next 4 years showed no duodenal ulcer recurred in *H. pylori*-negative patients (George *et al*., 1990). With the overwhelming research on the causal link between *H. pylori* infection and peptic ulcer disease, the Maastricht 2-2000 Consensus Report strongly recommended *H. pylori* eradication therapy for patients with gastric and duodenal ulcers (Malfertheiner *et al*., 2002).

Besides *H. pylori* infection, the use of non-steroidal anti-inflammatory drugs (NSAIDs) including aspirin, has also been considered to be a major risk factor implicated in the development of peptic ulcer disease (Huang *et al.*, 2002). In a study which evaluated the risk factors (*H. pylori,* NSAIDs and smoking) for peptic ulcer and related serious upper gastrointestinal (GI) events, the population-attributed risk associated with peptic ulcer is 48% for *H. pylori* and 24% for NSAIDs usage (Kurata & Nogawa, 1997).

Studies have also shown that occurrence of peptic ulcer have increased in *H. pylori* infected NSAIDs users (Kordecki *et al.,* 1997; Voutilainen *et al.*, 1998). In gastroduodenal mucosa, NSAIDs may augment acute inflammation caused by *H. pylori* where inflammation may then exacerbate the biochemical injury to these tissues caused by NSAIDs (McCarthy, 1991). However, the link between the use of NSAIDs and *H. pylori* infection is still a subject of clinical investigations, since conflicting results as to whether eradication of *H. pylori* would reduce the risk of peptic ulcer disease have been controversial (Chan *et al.*, 1997; Hawkey, 1999; Chan *et al.*, 2002). With regard to bleeding peptic ulcers, the report by Okan *et al.* (2003) showed a significant inverse relationship between *H. pylori* infection and NSAIDs usage. In their study population, the frequency of NSAIDs use in bleeding ulcers was shown to be high (79.2%). In contrast, the prevalence of *H. pylori* infection was lower in patients with bleeding. This negative interaction suggests a protective effect of *H. pylori* infection, lowering the risk of gastrointestinal bleeding in ulcer patients taking NSAIDs.

2.3.3 Gastric cancer

Research highlighting the association between gastric cancer and *H. pylori* has achieved plausible progress over the years. Substantial clinical and epidemiological evidence have shown that this gastric bacterium is a risk factor in the development of gastric cancer (Correa *et al*., 1990; Nomura *et al*., 1991; Huang *et al*., 2003; Wong *et al*., 2004). In 1993, the Eurogast Study Group reported data from 13 epidemiological studies which showed associations between *H. pylori* and cancer. The following year, the World

Health Organisation International Agency for Research on Cancer (IARC) designated *H*. pylori as a Class I (definite) carcinogen.

The association between *H. pylori* and cancer may be explained by 2 possible mechanisms: one is based on a carcinogenesis-promoting effect of the bacterium itself and the other is based on the establishment of a carcinogenic environment due to long-term infection. This long lasting infection may induce atrophic gastritis, which is considered to be the initial step in the gastritis-metaplasia-carcinoma sequelae in the stomach.

Phospholipase A2 of H. pylori was shown to cause epithelial cell membrane damage. In addition, the vacuolating cytotoxin (VacA+) of *H. pylori* constitutes increased risk for the development of peptic ulcer and gastric cancer. The reactive oxygen species (ROS) generated from the inflammation response by the host during an infection can also induce DNA damage with the accumulation of DNA mutations, thus leading to pathogenesis of gastric cancer (Obst et al., 2000).

The study by Uemura et al. (2001) convincingly showed the link between *H. pylori* and gastric cancer. It was a long term prospective study of 1246 *H. pylori* infected Japanese patients who had duodenal ulcers, gastric ulcers, gastric hyperplasia or non ulcer dyspepsia and 280 non-infected patients. After a mean followed up at 7.8 years (range 1-10.6 years), 2.9% of the infected while none of the uninfected patients developed gastric cancer.

Recent experimental animal model studies, in particular the development of cancer in Mongolian gerbils infected with *H. pylori* have provided direct evidence of the positive correlation of the bacterium with gastric cancer. Hirayama *et al.* (1996) reported that *H. pylori* could colonize the stomach of gerbils and induce gastritis 12 weeks after

inoculation. Gastric ulceration was observed at 24 weeks and intestinal metaplasia at 24 to 48 weeks after colonization. These pathological changes closely resembled the human pathology of *H. pylori* infection. Likewise, Shimizu and co-workers (2000) showed that eradication of *H. pylori* in *H. pylori* infected Mongolian gerbils reduced the incidence of adenocarcinoma. They commented that *H. pylori* eradication may be an effective way to prevent stomach cancer.

2.4 EPIDEMIOLOGY AND TRANSMISSION

2.4.1 Prevalence of *H. pylori* infection

The ubiquitous nature of *H. pylori* demonstrates no gender preponderance (Mitchell *et al.*, 1992; Goh *et al.*, 1997). The prevalence of *H. pylori* infection is not uniformly distributed as significant differences both within and between countries has been reported (Bazzoli *et al.*, 2001; Goh & Paraskthi, 2001; Robertson *et al.*, 2003). The general trend shows that the overall prevalence of infection in developed countries is lower than that in developing countries (Malaty *et al.*, 1996; Feldman *et al.*, 1998). High rates of infection have been attributed to low socio-economic status and high living densities (Moayyedi *et al.*, 2002). A cross sectional study which investigated the prevalence of *H. pylori* infection in Southern China showed an overall prevalence of 44.2% with a significantly higher rate in high population density urban areas (52.4%) as compared to 38.6% in rural regions (Mitchell *et al.*, 1992). Previous epidemiological studies have also documented an association of ethnic differences and the prevalence of *H. pylori* infection. Among adults in the United States, healthy Hispanic and black populations have seropositivity rates several folds higher than those of the non-Hispanic

white populations (Dehesa *et al.*, 1991). Singapore, a multi-racial country, has demonstrated differences in seroprevalence of infection among various races, with the highest in the Indian ethnic group (Kang *et al.*, 1997).

Natural acquisition of *H. pylori* infection occurs mainly in the early years of life (Boey *et al.*, 1999; Malaty *et al.*, 2002). Tindberg *et al.* (2001) in a cross sectional study found that 16% of children aged 10-12 years were infected. Similarly, Opekun *et al.* (2000) showed seroprevalence of *H. pylori* increased with age and was only 8.3% in very young children (6-11.9 months). During the past decades, the rate of *H. pylori* infection has dropped dramatically. Fujisawa *et al.* (1999) studied the changes in epidemiological pattern of *H. pylori* infection over the past 20 years in Japan. Sera of 1015 test subjects were collected and assayed for IgG against *H. pylori*. A decreasing pattern was evident with seroprevalence declining from 72.7% in 1974 to 39.3% in 1994. A drop in the prevalence of *H. pylori* has also been documented in Korean children of age younger than 10 years (Park *et al.*, 2001).

2.4.2 Probable sources and Routes of transmission

Source of Infection

So far, human is the principle source of *H. pylori,* although several animals which were considered as potential reservoirs such as domestic cats (Handt *et al.*, 1995) and rhesus monkeys (Handt *et al.*, 1997) have been exonerated. In addition to isolation of *H. pylori* from the animals, the bacterium has also successfully infected many laboratory animals (Karita *et al.*, 1991; Sturegard *et al.*, 1998; Koga *et al.*, 2003). Increased risk of infection with exposure to sheep was reported by Goodman *et al.* (1996) and the recovery

of *H. pylori* from sheep milk and gastric tissue (Dore *et al.*, 2001), suggests a possible zoonotic transmission.

Studies have claimed the domestic housefly as a vector of *H. pylori* infection (Grubel *et al.*, 1997; Vaira & Holton, 1998). However, Osato *et al.* (1998) demonstrated that *H. pylori* was not recovered from houseflies fed with human faeces either naturally infected or spiked with *H. pylori*. These results refuted the claim of housefly as a potential vector for transmission or a reservoir for *H. pylori* infection.

Water has also been implicated as a source of *H. pylori* infection. One of the first reports suggesting water as the source of infection is from Klein *et al.* (1991), which reported that children from homes using a municipal water supply were three times more likely to be infected than those whose homes had internal water sources. *H. pylori*-specific DNA was detected in environmental sources (Hulten *et al.*, 1998; Horiuchi *et al.*, 2001) but attempts to isolate the bacteria from water samples have been unsuccessful. The failure may relate to the morphology conversion of the spiral form to the dormant coccoid form under the adverse environmental conditions. Furthermore, experimental data from West *et al.* (1990) had demonstrated that this coccoid form is able to survive in river water for more than 1 year and subsequently be cultured.

Transmission

The mode of *H. pylori* transmission is still elusive and remains a subject of debate. However, the geographic and social patterns of *H. pylori* infection are consistent with human-to-human transmission via the faecal-oral or oral-oral route. Leung *et al.* (1999) published the findings on the isolation of *H. pylori* from contaminated vomitus. In the

study, 4 children presenting gastroenteritis associated vomiting were found to be seropositive for *H. pylori*. In one of the infected children's vomitus, *H. pylori* was isolated. In addition, Krajden *et al.* (1989) and Cellini *et al.* (1995) also reported the isolation of *H. pylori* from dental plague. Interestingly, comparison of the protein as well as the restriction endonuclease patterns of the isolates from the stomach biopsy and dental plaque displayed similar profiles (Cellini *et al.*, 1995). Attempts to culture *H. pylori* from faeces have been futile but in 1992, Thomas *et al.* cultivated the bacteria from faeces of 1 infected adult and 9/23 children from a Gambian village.

Employing the sensitive PCR method, *H. pylori* DNA was detected in a number of studies (Shuber *et al.*, 2002; Wisniewska *et al.*, 2002; Sicinschi *et al.*, 2003). Detection of DNA in the faeces lends credence to the faecal-oral route of transmission but it is essential to note that the presence of DNA does not imply that viable *H. pylori* is present.

Intrafamilial Transmission

Intrafamilial spread is implicated as a major route for acquisition of *H. pylori* infection. Familial aggregation of the infection has been shown in many studies (Oderda *et al.*, 1991; Dominici *et al.*, 1999; Miyaji *et al.*, 2000). The study by Rothenbacher and co-workers (1999) assessed the role of parental infection status in the transmission of *H. pylori* infection in 1221 pre-school aged children. The results provided strong evidence for a transmission pathway via parents to children.

Most of the epidemiological studies relied on the use of serology and urea breath test to define intrafamilial clustering. However, employing molecular techniques such as PCR based random amplified polymorphic DNA (RAPD) and restriction fragment length

polymorphism (RFLP) have further demonstrated the presence of closely related *H. pylori* strains among members of the same family thereby providing information about probable transmission of specific strain between individuals (van der Ende *et al.*, 1996; Han *et al.*, 2000; Roma *et al.*, 2003). An investigation by Goodman and Correa (2000) on rural Colombian children aged 2-9 years old, revealed that *H. pylori* infection transmitted most readily among siblings who were close in age, and most frequently from older siblings to the younger ones.

2.5 PATHOGENESIS OF *H. pylori* INFECTION

2.5.1 Adherence and Colonisation

Being a bacterial pathogen, *H. pylori* has to establish itself in the stomach following transmission. Adherence to the gastric epithelium is a crucial step in colonisation, a precursor of pathogenesis of *H. pylori*. All isolates expressed several putative colonization factors, including various adhesins, flagellar motility and urease (Eaton *et al.*, 1992; Hocker & Hohenberger, 2003).

H. pylori infection is a chronic infection and is considered unlikely that such infection remains with the absence of adhesin-host cell interactions (Evans & Evans, 2000). Adhesins are bacterial proteins, glycoconjugates or lipids that are involved in the initial steps of *H. pylori* infection and are important virulence factors. Presently, there is no consensus as to which *H. pylori* adhesins are most important *in vivo*. However, the best characterized adhesin is the blood group antigen-binding adhesion (BabA) which binds to difucosylated Lewis^{b} (Le^{b}) blood group antigens found on the gastric epithelial cells (Ilver *et al.*, 1998). Besides being an important adhesin, the presence of gene allele

babA2 was shown to be associated with *H. pylori* pathogenesis (Prinz *et al.*, 2001). Other adhesins included heat shock protein Hsp 60, *H. pylori* lipopolysaccaride (LPS), mucin binding proteins and neutrophil-activating protein (Valkonen *et al.*, 1994; Yamaguchi *et al.*, 1997; Namavar *et al.*, 1998).

H. pylori possesses 4-6 sheathed flagella and their presence appear to be essential in *H. pylori* infection. An isogenic non-motile mutant in the flagellar secretion apparatus component filQ resulted in a 30% reduction in adherence to human gastric cancer AGS cells (American Type Culture Collection no.CRL-1739) (Foynes *et al.*, 1999). In the *in vivo* study by Eaton *et al.* (1992), the non-motile *H. pylori* survived for only 6 days in infected piglets while the motile variant survived for a longer period of time (21 days). The study inferred that motility is necessary for full colonisation of gnotobiotic piglets by *H. pylori*.

The environment of the human stomach is usually within the pH range of 1-3.5. *H. pylori* is able to colonize the gastric epithelium in acidic conditions with the production of urease which metabolized urea to generate a neutral microenvironment. In the study by Eaton and Krakowka (1994), urease was shown to be essential for initial infection of normal mucosa in gnotobiotic piglets.

2.5.2 Determinants associated with pathological damage of the gastric mucosa

cag pathogenicity island and VacA cytotoxin

An important pathogenic factor is the cytotoxin associated gene (*cagA*). The *cagA* gene was identified as the marker for the presence of the *H. pylori* cag pathogenicity island (*cag* PAI). The immunopredominant protein encoded by this gene varies from

128 kDa to 140 kDa according to the number of copies of a 102 to 108 bp motif that is repeated with specific strains (Xiang *et al.*, 1995). Strains with the *cagA* gene were able to induce epithelial cells to secrete Interleukin 8 (IL-8) and cause mucosal inflammation (Orsini *et al.*, 2000). Several studies have then demonstrated a positive association of presence of *cagA* and peptic ulceration in adults (van Doorn *et al.*, 1998; Arents *et al.*, 2001). In contrast, studies from Asian countries such as Singapore, Japan, Korea and Hong Kong showed that *cagA* positive strains which were predominantly present were not associated with any specific clinical outcome (Yamaoka *et al.*, 1999; Zheng *et al.*, 2000; Wong *et al.*, 2001).

The *vacA* gene encodes a vacuolating toxin (~88 kDa) excreted by *H. pylori* that damaged a variety of mammalian cell lines *in vitro* and caused epithelial damage when the purified protein was administered intragastrically into mice (Leunk *et al*., 1988; Telford *et al.*, 1994). Allelic variation among *H. pylori vacA* occurs in the signal and middle region of the gene. The signal region exists as an s1 or s2 allele while middle region occurs as an m1 or m2 allele. *H. pylori* populations isolated from different geographical regions exhibit different distributions of *vacA* alleles (Yamaoka *et al.*, 1999; Zheng *et al.*, 2000). In North America and Western Europe, s1 *vacA* allele is associated with peptic ulcer disease (Atherton *et al.*, 1997; van Doorn *et al*., 1998). However, although s1 alleles predominate in Asia, no association of the *vacA* genotypes with any clinical manifestation was observed (Shimoyama *et al.*, 1998; Zheng *et al.*, 2000).

Lewis antigens

The O-antigen of lipopolysaccharide (LPS) in most *H. pylori* strains expresses the Lewis blood group antigens (Le) which are similar to those of the host (Kobayashi *et al.*, 1993). The ability of *H. pylori* to produce the Lewis antigens resembling those of the host appears to favour survival of the bacteria in the stomach by evading the immune response. In addition, this adaptation may also facilitate adherence of the bacteria to the gastric mucosa (Applemelk *et al.*, 2000). Another role of the presence of Lewis determinants is the possible involvement in pathogenicity. It was proposed that during an infection, *H. pylori* LPS could induce anti-Le antibodies which bind the bacteria and also the gastric epithelial cells, subsequently leading to tissue injury (Applemelk *et al.*, 1997). Interestingly, the study by Zheng *et al.* (2000) which examined 108 clinical isolates, demonstrated that although the presence of *cagA*, *vacA*, *iceA* genes were not associated with gastroduodenal diseases, the expression of 2 or more Le antigens was significantly higher in *H. pylori* isolated from patients with ulcers than from non-ulcer patients. A comparative study of expression of Le^X and Le^Y in *H. pylori* isolated from children and adults showed that expression of Le^Y was higher in adults than in children (Munoz *et al.*, 2001).

2.6 DIAGNOSIS OF *H. pylori* INFECTION

The detection of *H. pylori* infection is a primary requisite for diagnosis of gastroduodenal diseases related to this bacterium. In the paediatric population, duodenal ulcer is strongly associated with *H. pylori* infection and the risk of development of gastric cancer is relatively high if the infection is acquired at a young age (Blaser *et al.*, 1995a;

Huang *et al.*, 1999). In view of these critical issues, accurate diagnosis of *H. pylori* infection is a key step towards proper patient management. Two categories of diagnostic methods for *H. pylori* infection are defined: invasive and non-invasive.

Several factors such as the need to evaluate the sensitivity, specificity, positive and negative predictive value of a given test must be taken into account when selecting for a test. In addition, the age of patients being tested also has to be considered. At present, no single test can be absolutely relied upon to detect *H. pylori* colonization but if feasible, a combination of two tests is recommended (Laheij *et al.*, 2000). The European *Helicobacter pylori* Study Group (EHPSG) also recommend that two or more tests be performed as the gold standard in comparative studies (EHPSG, 1997).

2.6.1 Invasive tests

The invasive methods require gastric biopsy specimens obtained during gastroduodenoscopy. Presently, invasive biopsy tests include staining of histological samples, biopsy urease test, culturing of biopsy specimens and polymerase chain reaction.

Gastric biopsies

The most accurate method for detecting *H. pylori* in tissue is a combination of culture and histologic staining (Warthin Starry stain, Giemsa stains or hematoxylin & eosin stain) of the biopsy specimens obtained during endoscopy. With histologic staining, a histopathologist can document *H. pylori* infection and at the same time assess the atrophic changes in the stomach. However, the reliability of detecting *H. pylori* infection depends on factors such as the site, patching distribution of *H. pylori*, number and size of the biopsy specimens, as well as the stain used, not to mention the high level of expertise

in staining and visualizing the bacteria (El-Zimaity *et al.*, 1995; Woo *et al.*, 1996; El-Zimaity & Graham, 1999).

Despite the fact that culture will provide unequivocal evidence of the presence of *H. pylori,* successful recovery of the bacteria from biopsies will depend on transporting and processing of the samples, selection of media and culture conditions (Veenendaal *et al.*, 1993; Van der Hulst *et al.*, 1996, Piccolomini *et al.*, 1997; Grove *et al.*, 2001). This method enables the growth of fresh clinical isolates which can be tested for antibiotics susceptibility which can be useful in treatment management. The other salient advantage is to use the culture obtained for research, especially in molecular epidemiological study. In essence, obtaining the culture enables fingerprinting of the isolate and typing of the isolate with respect to the virulence genes.

Molecular biology techniques

Polymerase chain reaction (PCR) is particularly useful for molecular epidemiology and for fingerprinting *H. pylori* isolates. This technique has been used to genotype *H. pylori* in paraffin-embedded gastric biopsy specimens (Scholte *et al.*, 2002), which may be useful in the post-treatment period, to differentiate recurrence of infection from re-infection with another strain of *H. pylori* (Hildebrand *et al.*, 2001).

Recently, molecular techniques such as real time PCR and fluorescent in situ hybridisation (FISH) were evaluated for use in detection of *H. pylori* in gastric biopsy samples. The c*agA* and *vacA* genotypes were tested by melting curve using the real-time PCR and compared to the gastritis status and cell proliferation status (Ruzsovics *et al.*, 2001). The study by Russmann *et al.* (2001) demonstrated that this bacterial pathogen

was detected in 63 biopsy specimens as compared to 67 cultured positive specimens by FISH with rRNA-targeted fluorescence-labeled oligonucleotide probes specific for *H. pylori*.

2.6.2 Non-Invasive tests

Non-invasive tests obviate the need for endoscopy which can inflict physical stress on the patient. Moreover, invasive tests could yield possible false negative results due to the patchy distribution of the bacteria in the stomach (Pronovost *et al*., 1994). Clinical tests like the Urea breath test and serology test are well-established screening procedures which help to reduce the cost and workload of invasive endoscopy (Laheij *et al.*, 1998; Vaira *et al.*, 1999a). Given the special niche of *H. pylori*, attempts are continuously made to improve the non-invasive diagnostic tests.

Urea breath tests (UBT)

The UBT are easy, straightforward and accurate non-invasive tests for *H. pylori* infection. The test is based on the principle that in the presence of *H. pylori* urease activity, CO_2 is liberated from urea and produces ammonia to buffer its acidic environment. Ingestion of C-labelled urea results in the excretion of labelled CO_2 which can be detected in the expired breath. ^{13}C and ^{14}C UBT are semi-quantitative tests of active *H. pylori* infection (Debongnie *et al.*, 1991; Chang *et al.*, 2002).

^{13}C-UBT, containing the nonradioactive isotope, has high diagnostic accuracy in children (Delvin *et al.*, 1999). However, this method has its drawbacks:

1. It is difficult to perform in noncompliant children who are unwilling to ingest the ^{13}C-urea.

2. Age of children may make exhaled breath collection difficult.
3. Mental or physical disturbances may present further difficulty.

This was illustrated in the study by Imrie *et al.* (2001) which showed that borderline or false positive results occurred more frequently in children younger than 2 years compared with older children.

Serological tests

H. pylori elicits a local mucosal and a systemic antibody response. Serologic testing is based on the detection of anti-*H. pylori* IgG antibody in the patient's serum (Blecker *et al.*, 1995; Wang *et al.*, 2003). The simplicity and cost-effectiveness have enabled the serological tests to be widely employed for epidemiological research to assess the prevalence of *H. pylori* infection in various populations (Brown *et al.*, 2002; Chong *et al.*, 2003). The commonly employed serodiagnostic technique is the enzyme-linked immunosorbent assay (ELISA). The sensitivity and specificity of ELISA is dependent on the nature of the bacterial antigen preparation. Therefore, considerable research effort has been placed in search of a suitable antigen (Ho & Marshall, 2000). Commercial tests, the Pyloriset and the Helico-G use an acid extract of *H. pylori* antigen. The HM-CAP (Evans *et al.*, 1989) uses a mixture of high molecular weight *H. pylori* cell-associated proteins, consisting mainly of urease and cell wall adhesin.

The performance of serologic tests in diagnosis of treatment success is hampered by the lingering serological response after successful eradication therapy or spontaneous healing which will lead to false positive results. Furthermore, studies have shown that the cut-off values for children and adults may differ. Crabtree *et al.* (1991) showed that if the

adult cut-off value was used, 50% of the children with *H. pylori* gastritis would have been considered seronegative. Similarly, Sunnerstram *et al.* (1999) also recommended a lower cut-off value for children than for adults. It is clear that the choice of cut-off value is a mean of adapting the serological test to the patient population analysed. Hence, when children are tested for *H. pylori* antibodies, it is important to choose a method which has already been validated in the paediatric population.

Immunoblotting is highly sensitive and more specific than ELISA. It provides a full serologic profile of the immunogenic proteins that can be obtained from an individual. This method can be used to complement ELISA, especially when ELISA results are doubtful (Raymond *et al.,* 2000).

Stool antigen test (HpSA)

In 1998, the United States Food and Drug Administration (FDA) approved the use of a new enzyme immunoassay for the detection of *H. pylori* antigen in stool for diagnosis and eradication efficacy of *H. pylori* infection in adult population. *H. pylori* stool antigen test (HpSA) has been devised to detect the presence of *H. pylori* antigen by an ELISA method using monoclonal/polyclonal antibodies (Vaira *et al.*, 1999; Suzuki *et al.*, 2002).

Many studies have reported that HpSA test is useful for the initial diagnosis of *H. pylori* infection (Trevisani *et al.*, 1999; Ohkura *et al.*, 2000; Vakil *et al.*, 2000). However, its application in monitoring the efficacy of eradication therapy has been controversial as the assay can detect dead or partially degraded bacteria long after the successful eradication, leading to false positive results (Kabir, 2001). Nevertheless, a recent study by Tanaka *et al.* (2003) has shown that the HpSA test is a useful method for

initial diagnosis and after eradication therapy. They reported a high sensitivity (98.3%) and specificity (95%) prior to treatment. The sensitivity and specificity at the end of eradication therapy were 90% and 97.7%, respectively.

Accessibility and non-invasiveness have made this new test an invaluable diagnostic tool in the paediatric population. Data concerning reliability of the test in children has yielded satisfactory results (Husson *et al.*, 2000; van Doorn *et al.*, 2001; Koletzko *et al.*, 2003). In the study by Ni *et al.* (2000), involving 53 children, the performance of HpSA was evaluated and compared with 6 other diagnostic tests (Culture, biopsy urease test, histology, PCR, UBT and serology). The diagnostic accuracy of HpSA (sensitivity 92.6%, specificity 100%) was observed to be comparable to the other tests.

This non-invasive test was also found to be a useful method for post-treatment eradication testing of infection in children (Gosciniak *et al.*, 2003). In addition, the European *Helicobacter pylori* Study Group has recommended the stool antigen test and ^{13}C-UBT for diagnosis of the infection and eradication assessment in older children (Malfertheiner *et al.*, 2002).

Other non-invasive tests

Some studies have looked into the use of saliva and urine as possible non-invasive means to detect antibody to *H. pylori* infection (Miwa *et al.*, 1999; Luzza *et al.*, 2000; Yamamoto *et al.*, 2000). It was observed that the evaluation result for salivary assay was not satisfactory (Luzza *et al.*, 2000). However, the study by Yamamoto *et al.* (2000) had obtained a sensitivity of 92% and a specificity of 93.1% with the urine based ELISA kit.

Culturing of *H. pylori* from a gastric string which was to be swallowed by the individual has also been considered as a substitute for endoscopy biopsy (Ferguson *et al.*, 1999; Samuels *et al.*, 2000). The study by Samuels *et al.* (2000), demonstrated that *H. pylori* was isolated from 32/33 patients from a swallowed string coupling with the use of selective bacterial culture media. The string test also showed potential use as the culture obtained can be used for molecular epidemiological studies and the routine determination of antibiotic susceptibility prior to further therapy (Wang *et al.*, 2003).

2.7 GENOMIC AND PROTEOMIC STUDIES

2.7.1 *H. pylori* genome

H. pylori has a relatively small genome (~ 1.65 Mb) and is only about 1/3 the size of the *E. coli* genome. In comparison, the *H. pylori* genome was shown to possess 17% of species specific genes to the closely related organism *Campylobacter jejuni*, (Pennisi, 1999). Completed genomes of two unrelated *H. pylori* strains (26695 and J99) as well as the comparison of the two have been made available (Tomb *et al.*, 1997; Alm & Trust., 1999).

H. pylori 26695, sequenced by The Institute of Genome Research, was isolated in the mid 1980s in the United Kingdom from a patient suffering from gastritis. In contrast, *H. pylori* J99, an isolate from a duodenal ulcer patient in USA was sequenced in a collaborative study between Astra Zeneca R&D Boston (formerly Astra Research Center Boston) and Genome Therapeutics Corporation. Both genomes are displayed online at the following URLs: http//www.tigr.org/tigr-scripts/CMR2/CMRHomePage.spl and http://scriabin.astrazeneca-boston.com/hpylori/, respectively.

Comparison of the 2 genomes shows that *H. pylori* J99 is 24,036 bp shorter and contains 57 fewer predicted open reading frames than strain 26695. In addition, the analysis between the genomes of the two strains have also identified that each strain contains a set of genes (~6-7%) that are unique to each strain. Interestingly, almost one half of these genes were clustered in a single hypervariable region, namely the plasticity zone. In both J99 and 26695 genomes, almost 60% of the open reading frames (ORFs) were attributed with predicted functions (Alm & Trust, 1999).

Comparison of the two completed sequenced genomes has provided important information regarding the genetic heterogeneity. There are now evidence for some genetic loci such as *cagA* and *vacA* of the Western and Asian strains represented distinct lineages of *H. pylori* strains (van der Ende *et al*., 1998; Van Doorn *et al*., 1999).

2.7.2 Proteomics

"Proteome" was a term first introduced in 1995 (Wasinger *et al.*, 1995) to describe the protein complement of a genome. Proteomics is an emerging field of research that is essential to the interpretation of data evolving from the genomic studies. The technique of two dimensional polyacrylamide gel electrophoresis (2D PAGE) is at the heart of the proteomics research. 2D PAGE uses immobilised pH gradients in the first dimension to separate proteins on the basis of their p*I*, followed by SDS-PAGE run which subsequently characterises the proteins further according to their molecular sizes. Consequently, this 2D PAGE system can be loaded with a few milligrams of protein and separated with thousands of protein spots (Hanash, 2000; Gorg *et al.*, 2000). Proteomics can provide data on expression levels, subcellular location, post-translational modifications, protein

structure and domains. Furthermore, 2D PAGE also overcomes co-migrating proteins which appeared as a common band with 1D PAGE (Utt *et al.*, 2002).

The proteomics approaches have been used in the studies of *H. pylori*, attempting to link certain gel patterns to specific gastroduodenal pathologies (Enroth *et al.*, 2000) and elucidate proteins of potential diagnostic and vaccine candidates (McAtee *et al.*, 1998a, 1998b). With exhaustive proteomic characterization of *H. pylori*, Jungblut *et al.* (2000) and Bumann *et al*. (2001) had managed to resolve up to 1800 protein species using the high-resolution two-dimensional electrophoresis technique and identified more than 200 proteins. The results of protein identification were placed in the dynamic 2D PAGE database which is made publicly available at http://www.mpiib-berlin.mpg.de/2D-PAGE.

Comparative proteomics

In comparative proteomics, the proteins expressed by a given strain are quantitatively analyzed and compared to the proteome of another strain growing under similar conditions or same strain but grown in different conditions. For *H. pylori* to colonize and establish itself in the stomach mucosa, the bacteria must survive passage through the extreme acidity of the stomach to reach the mucous layer which is near neutral pH. Therefore, to understand the regulation of acid and base stress response in *H. pylori*, Slonczewski *et al.* (2000) investigated the effect of pH stress on protein profiles of *H. pylori* and had identified several proteins which were dependent on growth pH. Another comparative study was published by McAtee *et al.* (2001). In the study, the group examined the proteome of a metronidazole resistant strain (*rdxA* negative strain) upon exposure to sublethal doses of metronidazole. They revealed a total of 42 protein

spots which appeared to be differentially expressed and identified two upregulated proteins as alkylhydroperoxide reductase and aconitase B.

Immunoproteomics

Immunoproteomics is a useful approach for analysis of humoral responses, with the aim of screening and identifying immunogenic proteins which may contribute substantially to vaccine development and to the improvement of diagnostic techniques based on serology. This technique consists of systematic proteome analysis (2D PAGE), followed by immunoblotting with patient's sera. In *H. pylori* immunoproteome investigation, a number of studies revealed high variability of humoral recognition patterns among different patients and *H. pylori* isolates (Dunn *et al.*, 1989; Kimmel *et al.*, 2000; Jungblut *et al.*, 2000; Haas *et al.,* 2002).

In a recent study (Bumann *et al.*, 2002), mice were infected with *H. pylori* SS1 (a mouse-adapted strain originally isolated from a patient with peptic ulcer disease) and the proteins were resolved by 2D PAGE. Subsequently, the proteins were blotted, incubated with mouse sera and the immunogenic proteins were identified by matrix-assisted laser desorption ionization-Time of flight mass spectrometry (MALDI-TOF). When *H. pylori* immunoproteomes of the infected mice were compared to those proteomics data previously described as *H. pylori* antigens recognised by infected individual, a high agreement in the antigens recognized was observed. Hence, the study commented that murine *Helicobacter* model may be a valid model to screen antigens for vaccine development.

Difference gel electrophoresis (DIGE)

The reproducibility of the conventional 2D PAGE can be improved using DIGE, a newly emerging technique for comparative proteomics study. Different samples are labelled with mass and charge matched by spectrally resolvable fluorescent dyes, Cy2, Cy3 and Cy5, the pooled samples are then separated on the same 2D gel. Identical proteins existing in both pools will migrate to the same spot in the 2D gel, minimising the gel to gel variation over conventional 2D PAGE.

This technique has been applied to a model system study of the *E. coli* proteome after benzoic acid treatment (Yan *et al.*, 2002). Zhou *et al.* (2002) also employed this technique for identification of esophageal squamous cell cancer specific protein markers. They demonstrated that the DIGE technology has adequate sensitivity, high reproducibility and a wide dynamic range.

2.8 OTHER *Helicobacter* SPECIES

Since the identification of *Helicobacter pylori* in 1984, many novel *helicobacters* have been isolated from humans and animals. A growing number of other *Helicobacter* species which is shown in Table 2.2 are increasingly recognised as important human pathogens, most of which are likely acquired via zoonosis (Hua *et al.*, 1999b; Solnick, 2003).

In human, a helical bacterium different from *H. pylori* was first described in the gastric mucosa by Dent *et al*. in 1987, initially designated "*Gastrospirillum hominis*", which reflected its occurrence in human (McNulty *et al*., 1989). However, analysis of the

16S rRNA sequence showed that it is a *Helicobacter* species and has been provisionally named "*Helicobacter heilmannii*" (Solnick *et al.*, 1993).

In addition, *H. cinaedi*, *H. fennelliae*, *H. pullorum* and "*H. rappini*" have been isolated from cases of enteric disease, bacteremia and pneumonic illness. *H. cinaedi* and *H. fennelliae* have been isolated from the stools of homosexual patients with proctitis and proctocolitis (Totten *et al.*, 1985; Tee *et al.*, 1996).

H. hepaticus is found to be the aetiological agent of chronic hepatitis and associated with hepatocellular tumours in aged A/JCr mice (Fox *et al.*, 1994). Lin *et al.* (1995) have shown that the bile samples tested were positive for *H. pylori* DNA using the nested PCR method while Nilsson *et al.* (2000), showed that 12 of 37 serum samples from patients with chronic liver diseases had high antibody levels to *H. hepaticus* surface antigens even after adsorption with *H. pylori*. Thus, authors commented that some *Helicobacter* species might play a role in human liver diseases.

Neither the true niches occupied by this novel *Helicobacter* species is yet known nor is its spectrum of associated diseases fully defined. However, with the new developments in molecular techniques and improved isolation methods, it will not only better define the role of the *Helicobacter* species in human diseases but will also reveal novel species which are not currently identified. However, none of the *Helicobacter* species mentioned in Table 2.2 has been reported to be associated with infection in paediatric patients with dyspeptic symptoms.

Table 2.2 Other *Helicobacter* species isolated from humans (Adapted from Solnick, 2003)

Species	Clinical disease	Source(s)	Reference(s)
"*H. heilmannii*"	MALT lymphoma, peptic ulcer	Stomach	Solnick *et al.*, 1993; Trebesius *et al.*, 2001
H. felis	Gastroenteritis	Stomach	Lavelle *et al.*, 1994
H. cinaedi	Colitis, cellulitis	Faeces, blood	Totten *et al.*, 1985
H. fennelliae	Colitis	Faeces, blood	Quinn *et al.*, 1984; Fennell *et al.*, 1984
H. pullorum	Gastroenteritis	Faeces	Stanley *et al.*, 1994
H. canadensis	Gastroenteritis	Faeces	Fox *et al.*, 2000
H. winghamensis	Gastroenteritis	Faeces	Melito *et al.*, 2001
"H. rappini"	Colitis	Faeces	Romero *et al.*, 1988
H. canis	Gastroenteritis	Faeces	Burnens *et al.*, 1993

MATERIALS & METHODS

3.1 BACTERIAL STRAINS AND CULTURE CONDITIONS

H. pylori strain, RH 54 which was a clinical isolate from a local patient with healed duodenal ulcer and the standard strain NCTC 11637 were used in this study.

3.1.1 Fermenter cultures

H. pylori strain RH 54 was cultured in 10 ml brain heart infusion (BHI) broth containing brain heart infusion medium (Gibco) supplemented with 10% horse serum (Gibco) and 0.4% yeast extract (Oxoid) (Appendix A1.1) for 3 days at 37°C in a humidified CO_2 incubator (Forma Scientific) supplied with 10% CO_2. The 3 day old culture was subsequently reinoculated into 300 ml BHI broth and incubated for a further 3 days under similar culture conditions.

Following the incubation, cells were transferred to a 10L fermenter vessel (Bellco Biotechnology). The culture was incubated for 3 days to induce the active spiral form and for a prolonged period of 150 days to obtain the coccoid form according to the method as described by Ho and Vijayakumari (1993). Bacterial cells were tested for catalase, oxidase, urease activities (Appendix A2.1) and Gram stained to confirm identity of the culture. These bacterial cells were then harvested and used for the ELISA antigens preparation according to Khin and Ho (1994).

For subsequent genomic and proteomic studies (Section 3.11), *H. pylori* RH 54 and NCTC 11637 were kept for a longer incubation period of 184 days and 219 days, respectively.

3.1.2 Plate cultures

H. pylori strain, RH 54 was grown on chocolate blood agar plates (Appendix A1.2) and incubated for various time points (3, 5, 7, 14 & 30 days) at 37°C in a humidified CO_2 incubator supplied with 10% CO_2. The bacterial cultures were identified as *H. pylori* based on cell morphology by Gram staining (spiral shape for 3 day old culture & coccoid shape for prolonged cultures), positive catalase, oxidase and urease tests. Once confirmed, cells were harvested and used for subsequent studies.

3.2 SPIRAL AND COCCOID ANTIGEN PREPARATIONS

The antigens for serodiagnosis were prepared by a slight modification of the acid glycine extraction method as described by Khin and Ho (1994). Cells were harvested from the fermenter by centrifugation at 10,000 x g for 30 minutes at 4°C. The cell pellet was washed in sterile distilled water and the cell suspension was then centrifuged at 10,000 x g for 30 minutes at 4ºC. The supernatant obtained was discarded and wet weight of the pellet was determined. Cells were treated with 0.2 M acid glycine buffer (pH 2.2) at a concentration of 0.1g of cells (wet weight) to 2.5 ml of the buffer. The cell suspension was vortexed at room temperature for 30 minutes and centrifuged at 11,000 x g for 15 minutes at 4ºC, after which, cell debris was discarded.

The supernatant retained was neutralised to a final pH of 7.0. The neutralised supernatant was dialysed in dialysis tubing with MW exclusion limit at 12,000-14,000 daltons. The dialysis process was carried out overnight at 4°C against distilled water with gentle stirring. The dialysate was centrifuged at 3,000 x g for 10 minutes at 4ºC to remove any precipitates. This acid glycine extracted cell surface proteins constituted the acid

glycine extract (AGE) antigen which was employed in subsequent serologic testings. The protein concentration of the antigen was determined using Bio-Rad Protein Assay, based on the Bradford dye-binding procedure (Compton & Jones, 1985). All antigen preparations were stored at -20°C until use.

3.3 FAMILIAL CLUSTERS STUDY

3.3.1 Study population

Twenty one families comprising 72 subjects, age 1-73 years old who participated in a national serological survey conducted by the Ministry of Environment formed the basis of the study. The sera were kindly provided by the Ministry of Environment, Singapore. There were 2 families that were seronegative and thus served as negative controls for subsequent western blotting analysis. Of the remaining 19 families, all but one family had 2 or more members residing within the same household were seropositive for *H. pylori*. No comparison could be made for this family with only 1 seropositive member. Of the remaining 18 families, 3 families had three-tier relationships (grandparents, parents and children), 13 were of two-tiers (parents and children) and the remaining 2 families comprised only siblings.

3.3.2 Detection of antibody against the spiral antigen

IgG antibodies against *H. pylori* were determined using an in-house ELISA with the acid glycine extracted (AGE) antigen according to the method as described by Khin & Ho (1994). The spiral antigen was diluted to a final concentration of 5μg/ml with carbonate buffer (Appendix A3.1). The AGE antigen at 1μg (200 μl) of protein was

applied to each well in 96-well flat-bottomed microtiter plate (maxisorp, Nunc). Coated plates were incubated overnight at 4ºC.

After incubation, unbound antigen was removed and each well was refilled with 0.3 ml of serum diluent (Appendix A3.3) which served as the blocking buffer. Residual binding capacity of the plates was blocked at this step. The plates were incubated at 4ºC for an additional 24 hours before use.

The plates were brought to room temperature prior to use. The serum diluent was aspirated and wells were washed 3 times in wash buffer I (Appendix A3.4). Each washing step was about 3 minutes. After each rinse, residual liquid was removed by gently tapping the plates faced down onto several paper towels laying on the benchtop.

Cord blood sera which showed negative IgG antibodies against H. *pylori,* served as the negative control. This negative control and each test serum were diluted 100 fold in serum diluent. As for the positive control, it comprised pooled sera from patients who were histological positive for *H. pylori* and demonstrated strong IgG antibodies response against *H. pylori*. The positive control was diluted serially from 1:100 to 1:1600. Aliquot of 100 µl of each test serum and control sera were added to the coated wells. Each test serum was tested in triplicate. The plates were incubated at room temperature for 1½ hours to allow antigen-antibody binding. Following incubation, the plates were washed 3 times with wash buffer I. Peroxidase-labelled rabbit anti-human IgG (Dako) was used as the conjugate. It was diluted 1:25,000 with conjugate diluent (Appendix A3.5) and 100 µl of the diluted conjugate antibody was added into each well. The plates were left to incubate for another 1½ hours at room temperature. After incubation, plates were washed 3 times with wash buffer I, followed by 2 times with wash buffer II (Appendix A3.6).

Aliquot of 100 µl of substrate solution was added to each well and the plates were incubated in the dark for 15 minutes. The substrate solution comprised 0.03g of O-phenylenediamine hydrochloride (Sigma) and 30 µl of hydrogen peroxide (Merck) dissolved in 37.5 ml of phosphate-citrate buffer (Appendix A3.7). The enzymatic reaction was terminated with the addition of 2.5 M sulphuric acid. The optical density (OD) at 492 nm was determined in a microtitre plate reader (Labsystem Ascent). The absorbance value at OD_{492} was an average value of triplicate wells with the blank subtracted.

3.3.3 SDS-PAGE

Sample Preparation

The AGE proteins were concentrated using a speed vac concentrator (Heto Maxi dry lyo system). The concentrated proteins were dissolved in sterile distilled water to obtain a concentration of 30 µg proteins. The protein sample was mixed with 10 µl of 2X treatment buffer (Appendix A4.1) in an eppendorf tube and distilled water was added to make up a final volume of 20 µl. The tube was placed in a boiling-water bath for 5 minutes. The sample was then placed briefly on ice until use.

Homogeneous gel and electrophoretic run

A single percentage 12% separating gel and 4% stacking gel were prepared (Appendix A4.2-A4.8). This discontinuous system consisting of 2 gels with an upper stacking gel of pH 6.9 and a lower separating gel held at pH 8-9. The stacking gel concentrates the sample into thin bands which can accumulate at the interface between the 2 gels prior to separation. Using a pipettor with a long thin tip, 20 µl of sample was

loaded into each well. Wells which were not in use were loaded with 1X treatment buffer containing no sample. Electrophoresis was carried out using Bio-Rad Mini-Protean 3 Cell at 100 volts for 2 hours.

Coomassie Brilliant Blue G-250 staining

Coomassie Brilliant Blue G-250 which binds non-specifically to the proteins was used for staining the gels. This staining method, though less sensitive than silver staining (detection limit: 100 ng proteins) is widely use for its convenience. The staining protocol is adopted from Doherty *et al.* (1998).

After the electrophoretic run, the SDS-PAGE gel was soaked in the fixing solution containing 50% methanol and 2% phosphoric acid (85%). The gel was fixed overnight with gentle shaking. The gel was then washed 3 times with distilled water with 30 minutes incubation between each wash. Following which, the gel was incubated in incubation solution (34% methanol, 2 % phosphoric acid and 17% ammonium sulphate) for an hour. After 1 hour incubation, 660 mg of Coomassie Brilliant Blue G-250 was added to 1L of the incubation solution and further incubated with continuous shaking up to 3 days. After the gel was sufficiently stained, it was briefly rinsed with 25% methanol to remove the Coomassie Brilliant Blue G-250 colloids. The gel was then destained in distilled water. Several changes of distilled water enabled the proteins to be detected as blue bands on a clear background. Gel image was captured using the densitometer 710 (Bio-Rad) and analysis was performed using the Quantity One software (Bio-Rad).

Silver staining

Silver staining is a more sensitive method than the coomassie blue stain with a detection limit of 0.5ng of protein.

Staining of the gels was performed as described by Gharahdaghi *et al.* (1999). Briefly, gel was first soaked in fixing solution of 50% methanol and 5% acetic acid for at least 1 hour. After 3 changes of water at 20 minutes each, the gel was sensitized by incubating in 0.2% sodium thiosulphate (Fluka). This was followed by 2 quick washes of water for 1 minute each wash. The gel was immersed in 0.1% silver nitrate (Sigma) for 20 minutes. After incubation, gel was thoroughly rinsed with water and developed in 0.04% formaldehyde (37%) in 2% sodium carbonate (Merck). The reaction was terminated with 5% acetic acid. Image of gel was taken for subsequent analysis and storage using the densitometer GS 710 and Quantity One software (Bio-Rad).

3.3.4 Western blotting

Following SDS-PAGE, the gel was equilibrated in the transfer buffer (Appendix A5.1) for 10-15 minutes. The transblotting process was carried out using the semi-dry blotting apparatus (Bio-Rad). A few pieces of 3 MM Whatman filter papers were soaked in transfer buffer and placed on the bottom (anode) electrode plate of the blotting unit (Figure 3.1). The Immibilon polyvinylidene difluoride (PVDF) membrane was first prewetted in methanol for 20 seconds, followed by a quick rinse in distilled water. The treated membrane was then equilibrated in the transfer buffer and placed onto the filter papers. The whole cassette was completed with the equilibrated gel, few more pieces of wetted filter papers and the electrode plate (cathode) of the blotting device on top of the

stack. A pipette was used to gently roll over the sandwich to remove air bubbles which would interfere with the electroblotting. A constant current of 150 mA (Model 200/2.0 power supply, Bio-Rad) was applied for about an hour.

The efficiency of the transfer was accessed by the transfer of the prestained molecular weight marker onto the membrane or using a Ponceau S stain to visualize the protein patterns on the membrane after blotting. The blot was then blocked for 1 hour with agitation on a belly dancer shaker (Stovall Life Sciences Inc) in 5% skim milk in PBS Tween-20 (blocking solution) (Appendix A5.2). After blocking, the blot was washed with wash buffer (Appendix A5.3) to remove any unbound blocking agent. The membrane could be cut into several strips and placed into wells of a staining tray with diluted primary antibodies (patient's sera diluted 1:200 in 1% BSA/PBS Tween-20). The tray was covered and incubated overnight at room temperature with gentle shaking on the shaker.

After incubation, the strips were washed with wash buffer and incubated with secondary antibody (horse radish peroxidase labelled rabbit anti-human IgG) diluted 1: 800 in 1% BSA/PBS Tween-20. The strips were incubated for 1½ hours. Subsequently, detection was achieved using the substrate, 4-chloro-1-napthol (Sigma). The substrate solution (Appendix A5.4) was prepared by dissolving 60 mg 4-chloro-1-napthol in 20 ml 100% cold methanol. This solution was then added to 100 ml of PBS buffer with 60 μl of 30% hydrogen peroxide added prior to use. Strips were incubated in this substrate solution in the dark till bands developed. The reaction was stopped by rinsing with distilled water repeatedly. The strips were air-dried and imaged using a GS 710

densitometer (Bio-Rad). Image analysis was carried out using the Quantity One software (Bio-Rad).

3.3.5 Statistical Analysis

Cohen's kappa statistical analysis was used to assess the agreement among the seropositive members in each household. According to Fleiss (1981), values greater than 40% are indicative of moderate levels of agreement chance. Values exceeding 75% strong agreement above chance. The protein weight of each band was calibrated and the degree of agreement was determined statistically by the statistical program SPSS version 10. In this study, we adopt a kappa value of ≥ 40% to be indicative of similarity between the compared immunoblots.

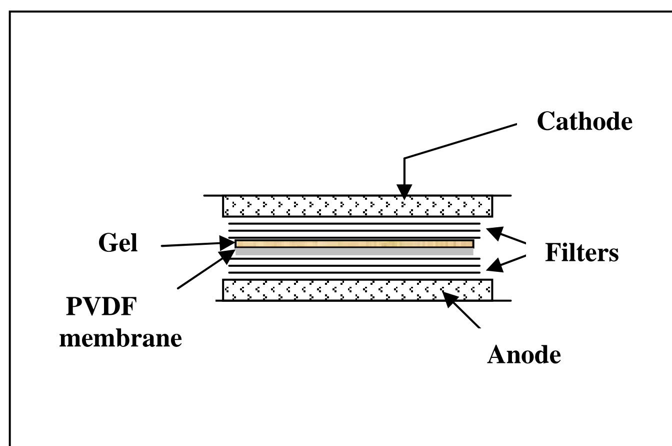


FIG 3.1 Gel membrane assembly for semidry electrophoretic transfer cells.

3.4 STUDY ON PAEDIATRIC PATIENTS

3.4.1 Cut-off value for ELISA

Many reports have highlighted the need of an ELISA cut-off for paediatric patients with dyspeptic symptom (Crabtree *et al*., 1991; Sunnerstam *et al*., 1999; Kindermann *et al*., 2001).

In this study, the cut-off value for ELISA was based on a pre-evaluated comparative study of 114 local paediatric patients comprising 15 culture/histology positive and 99 histology negative samples. Receiver operating characteristic (ROC) analysis was performed to determine the ideal cut-off point for the serological test.

3.4.2 Study population

In this study, a total of 489 consecutive children (mean age of 8.5 ± 3.3 years) with epigastric pain formed the study population. They were out-patient referrals to the paediatric gastroenterology clinic at the National University Hospital, which is a tertiary referral centre in Singapore. All children had been having epigastric pain for at least 3 months, and had consulted their own general practitioners or paediatricians prior to referral to the specialist gastroenterology clinic. The patient population consisted of 263 girls and 226 boys. All the symptomatic children did not receive any antibiotics within 4 weeks of the study. Blood sample was collected from each individual. Serum was separated from the blood, aliquoted and kept at -20ºC until use. The control group comprised 599 school children (age 9 ± 0.5 years) in the eastern part of Singapore who participated in a seroepidemiological survey conducted by the Ministry of the

Environment. The sera for this control group were kindly provided by the Ministry. Informed consent was obtained from parents of the children.

3.4.3 *H. pylori* antibody determination

The presence of immunoglobulin antibodies (IgG) against spiral (3 day old culture) and coccoid (150 day old culture) forms of *H. pylori* in all the sera was determined using in-house ELISA with antigen prepared from *H. pylori* RH 54 clinical isolate (Khin & Ho, 1994). The procedure was as described in Section 3.3.2. Samples were tested in triplicate.

3.4.4 Western blot profiling of representative sera

Sera from 5 children who were positive for *H. pylori* infection by either histology or culture and 4 who were negative for the infection but experienced epigastric pain were randomly selected for western blot. The procedures were as described in Section 3.3.4. In this study, both the spiral antigen (3 day old culture) and the coccoid antigen (150 days old culture) were employed. Comparison of the antibody profiles was performed for the two groups and between the two antigens.

3.4.5 Statistical evaluation

Statistical analysis was performed using the statistical software SPSS version 10 (Scientific Package for Social Sciences, Chicago, USA). The χ^2 test and Fisher's exact test were used to compare the proportions between groups. The level of significance was set at a $P < 0.05$.

3.5 CHARACTERIZATION OF *H. pylori* ISOLATES FROM PAEDIATRIC PATIENTS WITH DYSPEPTIC SYMPTOMS

3.5.1 Clinical strains from paediatric patients

A total of 15 clinical *H. pylori* strains isolated from paediatric patients with epigastric pain were randomly selected from our culture stock for the characterization study. These isolates were reconfirmed as *H. pylori* by gram staining and biochemical tests.

These strains were isolated from the gastric antral biopsies of paediatric patients with epigastric pain, referred for gastroduodenoscopy at the Endoscopy Centre in the National University Hospital. Informed consent was obtained from parents of the paediatric patients.

3.5.2 Identification of *H. pylori*

The isolates were grown on the chocolate blood agar plates for 3 days at 37°C in a humidified CO_2 incubator. *H. pylori* appeared as characteristic water droplet-like colony of 1-2 mm in diameter.

3.5.2.1 Gram stain

Gram staining of the culture showed that *H. pylori* is a gram negative spiral shaped bacteria and motile spiral under phase contrast microscopy.

3.5.2.2 Enzymatic tests

Urease Test

A small inoculum of the bacterial isolate was added to the urea reagent (Appendix A2.1). A positive reaction was indicated by the colour change of the reagent from yellow to bright pink within 24 hours.

Catalase Test

A colony of the bacterial isolate was smeared onto the glass slide and a drop of 3% hydrogen peroxide (Merck) added to the smear. A positive result was characterized by the presence of effervescence.

Oxidase Test

A strip of filter paper was dipped into 1% oxidase reagent, tetra-methyl-p-phenylenediame dihydrochloride (Sigma). With a sterile toothpick, bacterial colony was transferred onto the reagent soaked filter paper. Typical *H. pylori* shows a positive purple colour patch developed at site of smear.

3.6 DETECTION OF VIRULENCE GENES

3.6.1 Extraction of genomic DNA

The phenol-chloroform extraction method (Hua *et al.,* 1996)) was used to obtain DNA from the isolates. Genomic DNA was prepared from freshly harvested 3 day old plate culture. The bacterial cells were transferred to an Eppendorf tube with TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0) (Appendix A6.1). The suspension was centrifuged at 8,000 x g for 10 minutes in a microfuge (Sovall Biofuge) and gently washed

once with TE buffer. The pellet was suspended with 800 µl TE buffer and 100 µl of 10 mg/ml lysozyme (Sigma Chemical Co.) and incubated at 37°C for 30 minutes. The bacterial suspension was lysed with 100 µl of 10% sodium dodecyl sulphate for another 30 minutes at 37°C. This was followed by the addition of 5 µl of 10 mg/ml proteinase K (Boehringer Mannheim GmbH.) to the mixture and incubated at 56°C for 1 hour. Nucleic acids were extracted twice with equal volume of phenol and once with equal volume of chloroform. DNA was precipitated by addition of one volume of absolute ethanol and 20 µl of 3M sodium acetate at -20°C overnight before centrifuging at 12,000 x g for 10 minutes in a microfuge. The DNA pellet was washed once in 70% ethanol. Thereafter, it was vacuum dried with a speed vac concentrator (Heto Maxi dry lyo system) and resuspended with 200 µl of sterile distilled water. The concentration and quality of each DNA sample was estimated by the ratio of absorbance at OD 260/280. The DNA was kept at -20°C until use.

3.6.2 Identification of virulence genes using PCR

The presence or absence of various virulence genes (*cagA, vacA, iceA* alleles) was carried out using PCR with specific primers presented in Table 3.1 and Table 3.2. Two sets of primers were used for each gene examined. The PCR reaction was performed with a 50 µl volume which consisted of 50 ng of *H. pylori* genomic DNA, 1X PCR buffer (10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl and 0.1% Triton X-100), 0.2 mM each deoxynucleotide, 50 pmole of each specific primer and 1U of DyNAzyme DNA polymerase (Finnzymes). The amplification cycle was performed in a GeneAmp PCR system (Perkin-Elmer) thermocycler. PCR conditions were an initial cycle of 94°C for

5 minutes, 50°C for 1 minute, 72°C for 1 minute, followed by 40 cycles of 94°C for 1 minute, 50°C (annealing temperature) for 1 minute and 72°C for 1 minute. Final extension was performed at 72°C for 5 minutes to ensure full extension of the PCR products.

PCR reaction for one of the *babA* gene alleles, *babA2* was also performed using the specific primers as listed in Table 3.1. The reaction mixture of a final volume of 50 µl comprised 40 ng genomic DNA, 1X PCR buffer, 0.2 mM each deoxynucleotide, 10 pmole of both forward and reverse primers and 1U of DyNAzyme DNA polymerase. The PCR reaction was as described for the *cagA, vacA and iceA* genes. However, annealing temperature was set at 52°C instead of 50°C.

3.6.3 Agarose gel electrophoresis

The PCR amplification products were run on 1% horizontal agarose gel in TAE buffer (Appendix A6.2). Each well was loaded with 10µg sample with 1X DNA gel loading buffer (Appendix A6.3). The gels were stained with ethidium bromide and transilluminated with UV light to visualise the PCR products. The size of the amplification product was used to confirm the presence of the gene. The image was captured using Kodak EDAS 290.

3.7 DNA FINGERPRINTING

3.7.1 RAPD fingerprintings of various clinical strains

A universal oligonucleotide primer 773 (5'-AAGAGCCCGT-3') as described by Akopyanz *et al*. (1992) was chosen for PCR-RAPD and the amplification condition was

carried out at low stringency (Hua *et al.*, 1998b). Briefly, the reaction mixture consisted of 50 ng genomic DNA, 2 mM $MgCl_2$, 20 pmol primer 773, 1X PCR buffer, 1U DyNAzyme DNA polymerase and 200 mM each deoxynucleotide in a final volume of 25 µl. PCR reaction was performed with a 5 minute preincubation at 94°C, followed by 39 cycles of denaturation at 94°C for 1 minute, annealing at 36°C for 1 minute and extension at 72°C for 2 minutes. PCR products were examined by electrophoresis on 1% agarose gels.

3.7.2 RAPD profiles of clones

To access whether multiple-strain colonization occurs in local paediatric patients, 5 single colonies from each isolate were picked from the primary growth. Five randomly selected isolates were used in this clonal study. DNA was isolated from the individual clone by the phenol-chloroform extraction method. RAPD profile of each clone was obtained by running the PCR reactions with the universal primers with conditions as described in section 3.7.1. DNA fingerprints for the 5 different clones from individual isolate were obtained when the PCR products were run on a 1% agarose gel. In addition, each clone was also tested for the prevalence of the virulence genes by PCR.

3.8 Lewis antigens expression of *H. pylori* Isolates

3.8.1 Primary isolates

The expression of Lewis (Le) antigens was determined as described by Simoons-Smit *et al.* (1996) using ELISA with whole *H. pylori* cells as the antigen. *H. pylori*

TABLE 3.1 PCR Primers used in this study

Gene region amplified	Primer sequence (5' ⟶ 3')	PCR product (bp)	T_a (^0C)	Reference
26kDa	TGGCGTGTCTATTGACAGCGAGC CCTGCTGGGCATACTTCACCAAG	298	50	Hammar (1992)
cagA	AATACACCAACGCCTCCAAG TTGTTGCCGCTTTGCTCTC	400	50	Lage (1995)
vacA	GCTTCTCTTACCACCAATGC TGTCAGGGTTGTTCACCATG	1160	50	Xiang (1995)
iceA1	GTGTTTTTAACCAAAGTATC CTATAGCCAGTCTCTTTGCA	246	50	van-Doorn (1998)
iceA2	GTTGGGTATATCACAATTTAT TTGCCCTATTTTCTAGAGGT	229/334	50	van-Doorn (1998)
babA2	AATCCAAAAAGGAGAAAAAGTATGAAA TGTTAGTGATTTCGGTGTAGGACA	831	52	Gerhard (1999)
ureA	GCCAATGGTAAATTAGTT CTCCTTAATTGTTTTTAC	411	50	Peek *et al.* (1995)
ureC	AAGCTTTTAGGGGTGTTAGGGGTTT AAGCTTACTTTCTAACACTAACGC	294	50	Labigne *et al.* (1991)
Random hexamers	NNNNNN			

T_a (annealing temperature)

TABLE 3.2 Primers for *vacA* typing

Gene region amplified	Primer sequence (5'⟶ 3')	PCR product (bp)	T_a (^{0}C)	Reference
*s1a**	TCTYGCTTTAGTAGGAGC	212	52	Yamaoka *et al* (1999)
	CTGCTTGAATGCGCCAAAC			
s1b	AGCGCCATACCGCAAGAG	187	50	Atherton *et al* (1995)
	CTGCTTGAATGCGCCAAAC			
*s1c**	CTYGCTTTAGTRGGGYTA	213	52	Yamaoka *et al* (1999)
	CTGCTTGAATGCGCCAAAC			
s2	GCTAACACGCCAAATGATCC	199	52	Atherton *et al* (1995)
	CTGCTTGAATGCGCCAAAC			
m1	GGTCAAAATGCGGTCATGG	290	53	Atherton *et al* (1995)
	CCATTGGTACCTGTAGAAAC			
m2	GGAGCCCCAGGAAACATTG	352	52	Atherton *et al* (1995)
	CATAACTAGCGCCTTGCAC			
m1T	GGCCACAATGCAGTCATGG	290	50	Wang *et al* (1998)
	CTCTTAGTGCCTAAAGAAACA			
m1Tm2	GGCCACAATGCAGTCATGG	300	54	Wang *et al* (1998)
	CATAACTAGCGCCTTGCAC			

T_a (annealing temperature)

* Y is C or T and R is A or G

isolates grown for 3 days on chocolate blood agar plates were harvested in sterile phosphate buffered saline (PBS), pH 7.4. Bacterial suspension of OD_{600} O.5 (~7.5 x 10^7) was prepared and 100 µl of the whole cell suspension was coated per well in a 96-well flat bottomed microtiter plate (polysorp, Nunc). The coated plates were incubated overnight at room temperature. The plates were washed 3x with PBS containing 0.05% Tween 80 (PBST). Murine monoclonal antibodies (Mabs) against Le^x, Le^y, Le^a and Le^b (Signet Laboratories) were used as primary antibodies. An aliquot of 100 µl of Mabs (100 µg/ml) was added and incubated overnight at room temperature. After incubation, the plates were washed with PBST. The secondary antibody, goat anti-mouse immunoglobulins conjugated with horse radish peroxidase (Dako) was diluted 1:1000 with PBST, supplemented with 0.5% goat serum (Dako). An aliquot of 100 µl of the secondary antibodies was added to each well and incubated at 37°C for 2 hours with gentle shaking and washed. After which, colour developed with the addition of 100 µl of developing solution containing 1 mg/ml *ortho*-phenylenediamine dihydrochloride (Sigma) in phosphate citrate buffer, pH 5.4 (0.1 M $C_6H_8O_7H_2O$ and 0.2 M $Na_2HPO_4H_2O$) and 0.5 µl of H_2O_2 (30% [vol/vol]) per ml. The plates were then incubated in the dark for half an hour before the addition of 50 µl of 10% (vol/vol) H_2SO_4 to each well to stop the reaction. The colour change was measured at 492 nm with Labsystem Ascent microtitre plate reader (Finland). The optical density values > 0.2 was considered positive reactions (Zheng *et al*., 2000).

The specificity of the Mabs was validated by their ability to recognise the respective antigen from a panel of synthetic Le antigens (Le^x, Le^y, Le^a and Le^b) (IsoSep).

3.8.2 Aging cultures

H. pylori RH 54 and NCTC 11637 were grown on chocolate blood agar plates and harvested over a period of 3-219 days at different time points. The expression of Lewis antigens (Le) was determined using an ELISA as described in Section 3.8.1. Each sample was tested in duplicate and the mean of the ODs was used for analysis.

3.9 ANTIMICROBIAL SUSCEPTIBILITY TESTS

H. pylori clinical isolates were tested for susceptibility to the 4 commonly used antimicrobial agents in the treatment of *H. pylori* infection. These were tetracycline, amoxicillin, metronidazole and clarithromycin. The susceptibility test was carried out using the disk diffusion and Epsilometer test (E-Test).

3.9.1 Disk diffusion method

Isolates were grown on chocolate blood agar for 3 days, harvested and was suspended in 0.85% sterile saline. An inoculum containing ~10^8 CFU/ml in sterile saline was spread with a cotton swab onto the surface of chocolate blood agar and left to dry on the bench for about 5 minutes. The following antibiotic disks (Oxoid) were placed onto each agar plate: tetracycline (30μg), amoxicillin (10μg), metronidazole (5μg) and clarithromycin ((15μg). All plates were then incubated in microaerophilic atmosphere (10% CO_2) at 37°C for 3-4 days. The zone of inhibition was measured (in mm). The zone size breakpoints are listed in Table 3.3.

3.9.2 Epsilometer test (E-test)

E-test is a quantitative technique for determining the antimicrobial susceptibility of both non-fastidious Gram negative and Gram positive aerobic bacteria. E-test strip (AB Biodisk) is a strip which one side of the strip is calibrated with an MIC reading scale in µg/ml. Whilst the other side is the predefined exponential gradient of antibiotic, dried, stabilized and is immobilized on the surface of the strip. All antimicrobial agents were tested at concentration ranging from 0.016 to 256 µg/ml. The agar plates were inoculated by confluent swabbing of the surface with the bacterial inoculum and left to dry on the bench at room temperature for 5 minutes. The E-test strips were then applied onto the surface of each agar plate. The plates were incubated at 37°C under microaerophilic conditions. After the 72 hours incubation period, the MIC (minimium inhibitory concentration) value was read as the intersection between the inhibition ellipse edge and the E-test strip. Table 3.4 provides the MIC values for resistant strains when subjected to the antibiotics.

3.10 ELECTRON MICROSCOPY AND FLOW CYTOMETRY

3..0.1 Raising of antibodies against spiral and coccoid antigens

Immunization to produce antibodies

New Zealand white rabbits weighing ~1.2 kg were used in this immunization protocol. Spiral antigen (RA 101) /coccoid antigen (BCD 23) of 250 µg was mixed with equal volume of complete freunds adjuvant (CFA; Sigma #F5881). The CFA/antigen mixture was drawn into a 1 ml syringe. With a double-ended locking hub connector, the mixture was mixed till it formed an emulsion. Prior to immunization, pre-immune blood

sample was collected in a 15 ml centrifuge tube by bleeding the marginal veins of the rabbit. The CFA antigen emulsion was injected into the rabbits subcutaneously. The rabbits were bled 14 days after the priming immunization and blood samples were collected. This was referred to as the immunised blood sample. Subsequently, booster immunizations using incomplete freunds adjuvant (IFA; Sigma #F5506) (instead CFA) were administered 4 weeks after the priming immunization. Further booster immunizations were given at 2 week intervals. Immunised blood was collected prior to each subsequent booster immunizations. The blood samples collected were allowed to incubate at 37ºC for an hour and then 4ºC overnight until clot was formed. Serum was separated by centrifugation and the antibody titre was assayed by ELISA. The serum was stored at -20ºC until use.

Purification of antibodies

Protein A Sepharose CL-4B affinity column (Amersham biosciences) was used to isolate IgG from the serum. IgG binds Protein A Sepharose CL-4B at neutral pH and physiological strength. Thus, the serum was diluted with 50 mM Tris buffer, pH 7.0 and loaded into the column. The IgG was eluted with 0.1 M glycine buffer, pH 3.0 and to preserve the acid labile IgG, 100 µl of 1 M Tris-HCl, pH 9.0 was added to neutralize the eluted fractions.

TABLE 3.3 Zone diameter breakpoints for *H. pylori* (Disk Diffusion)

Antibiotics	µg/disk	Zone size breakpoints (mm) (resistance)	References
Amoxicillin	10	< 17	Iovene *et al* (1999)
Clarithromycin	15	< 30	Midolo *et al* (1997)
Tetracycline	30	< 19	Midolo *et al* (1997)
Metronidazole	5	< 15	Decross *et al* (1993)

TABLE 3.4 MIC breakpoints for *H. pylori* (E-Test)

Antibiotics	MIC breakpoints (mg/L) (resistance)	References
Amoxicillin	≥ 2	King (2001)
Clarithromycin	≥ 2	King (2001)
Tetracycline	≥ 4	King (2001)
Metronidazole	≥ 8	King (2001)

3.10.2 Immunogold labelling for Transmission Electron Microscopy (TEM)

H. pylori strain RH 54 of different ages (3 day and 30 day old) were harvested and washed 3X with phosphate buffered saline (PBS), pH 7.4 (Appendix A3.2) for 5-10 minutes at each change. The cells were then fixed for 20 minutes in 4% paraformaldehyde + 0.1% glutaraldehyde with 0.5% Triton-X in PBS. This was followed by another 3 washes with PBS. Sample was incubated in 0.05 M glycine-PBS for 15 minutes before 3 washes with PBS. The fixed cells were blocked with 0.5% bovine serum albumin (BSA)-PBS buffer. Following that, cells were incubated overnight with the respective purified 1:20 diluted spiral/coccoid antibodies at 4ºC. All antibody dilutions and subsequent washes were carried out using the BSA-PBS buffer. The cells were washed 3X with BSA-PBS buffer and bound antibodies were localized by incubating the cells for 2 hours at room temperature with 1:20 diluted 10nm gold-conjugated protein A (Sigma). This was followed by 2 changes of PBS with 0.5% BSA and another 2 washes with PBS without BSA. The immunogold labelled cells were post-fixed with 2% glutaraldehyde in PBS at room temperature for 2 hours. After which the cells were rinsed 3X with BSA-PBS buffer and left overnight at 4ºC. The next step was to post-fix the cells with 2% osmium tetroxide (OsO_4) in PBS for about an hour. After 3 washes in BSA-PBS buffer, the cells were dehydrated in an ascending ethanol series (50%, 75% and 95%) at room temperature for 15 minutes at each change. This was followed by 3 changes of absolute ethanol for 20 minutes each. Subsequently, cells were infiltrated with mixtures of ethanol and embedding media, Low Viscosity Epoxy Resin (LVER) by passing through 3 changes of the mixtures of various proportions:

1st change- 7 parts ethanol + 3 parts LVER for 30 minutes

2nd change- 5 parts ethanol + 5 parts LVER for 30 minutes

3rd change- 3 parts ethanol + 7 parts LVER overnight

The fixed cells were incubated overnight in the last change of mixture (3 parts ethanol + 7 parts LVER). The following day, cells were then passed through 3 changes of freshly prepared LVER. These cells were left in media for an hour in between each change. During the last change, cells were transferred to beem capsules for embedding and polymerisation at 65ºC for 16-18 hours. Ultra-thin sections were cut using a microtome (Ultra-cut E, Leica) and contrasted with uranyl acetate and lead citrate. The sections were then viewed under the Philips 208S transmission electron microscope at the Electron Microscopy Unit, NUS.

3.10.3 Flow cytometry analysis

The specificity of the antibodies raised against spiral and coccoid antigens to the *H. pylori* cells at various time points was evaluated using the flow cytometry. *H. pylori* culture of different ages ranging from 3-184 days were used. At each time point, *H. pylori* culture was harvested. Cell pellet was suspended in PBS to OD_{600} of 0.5. After the OD adjustment, 1ml of the bacterial suspension diluted in PBS was centrifugation at 7,700 x g for 5 minutes. The supernatant was decanted and pellet loosened by gentle vortexing after the addition of 100 µl of the respective antibodies (spiral/ coccoid) diluted in 0.2% BSA-PBS (w/v) to a final concentration of 1 µg/ml. The mixture was incubated on ice for 30 minutes. Following incubation, the mixture was centrifuged at 7,700 x g for 5 minutes and the supernatant was discarded. The pellet was then resuspended in 0.2% BSA-PBS

and mixed with 100 µl of anti-rabbit IgG FITC (fluorescein isothiocyanate) conjugate (1:50 dilution). The mixture was incubated in the dark on ice for another 30 minutes before it was centrifuged. The pelleted bacteria labelled with FITC were resuspended in 300 µl PBS in FACS tubes. Bacteria pellets without FITC resuspended in 300 µl PBS were used as negative control. Flow cytometry analysis of stained cell suspension was performed on the flow cytometer, Coultier Elite ESP (Beckman Coultier). A total of 10,000 gated events were collected and analysis of the data was carried out by using the winMDI software version 2.8.

3.11 GENOMICS AND PROTEOMICS STUDIES

3.11.1 RAPD profiles of RH 54 and NCTC 11637 at various time intervals

At each time point (3, 5, 7, 14, 30 days), *H. pylori cells* were harvested and washed with sterile PBS. For induction of the coccoid form of *H. pylori*, prolong culture of both strains were prepared by growing the cultures in the fementer vessels as described in Section 3.1.1. Strain RH 54 was grown for a period of 184 days while NCTC 11637 was grown for 219 days. Bacterial cells were harvested and genomic DNA was extracted using phenol-chloroform method (Hua *et al*, 1996). The DNA profiles for each time point were generated by PCR-RAPD using the single short oligonucleotide primer 773 (5'-AAGAGCCCGT-3'). The PCR products (10 µl) were analyzed by electrophoresis on a 1% agarose gel containing ethidium bromide and profiles were captured using Kodak EDAS 290.

3.11.2 Putative virulence genes in aging cultures

The DNA of the ageing cultures (a period of 3 days to 184/219 days) was extracted. PCR amplification of the various virulence genes were performed using the primers listed in Table 3.1 and with respective conditions as described in Section 3.6.2.

3.11.3 Gene expression in aging cultures

Total RNA Preparation

Total RNA at the various time intervals was prepared using a commercially available total RNA-extraction kit (RNeasy Mini, Qiagen). Firstly, bacteria (~1 X 10^9) at various time intervals were harvested by centrifuging at 5,000 x g for 5 minutes at 4°C. The cell pellet was retained and subsequent extraction process was performed at 20-25°C. The cell pellet was resuspended in 100 µl of lysozyme containing TE buffer (400 µg/ml) and vortexed for 5 minutes. The extraction steps were carried out according to the protocol provided by the supplier. After isolation of the total RNA, it was treated with DNase I (Sigma) by incubation at 37°C, followed by inactivation of the reaction mixture at 95 ºC for 5 minutes. In order to exclude any amplification bands resulted from residual DNA, an aliquot of the DNase mixture was subjected to PCR using primers for *ureC* gene. The prepared RNA was frozen at -80°C until use.

Preparation of cDNA

The RNA (2 µg) was transcribed to cDNA using the random hexamers as primers (Table 3.1). The mixture was incubated at 70ºC for 10 minutes and placed on ice for 2 minutes. The mixture was incubated with 1X reverse transcriptase buffer (Promega),

1 mM deoxynucleotides and 40U of RNasin ribonuclease inhibitor (Promega) at 25ºC for 5 minutes and then 10 U of Avian Myeloblastosis Virus Reverse Transcriptase (AMV RT) (Promega). The sample was placed at 25ºC for 10 minutes and then at 37ºC for an hour. The reaction mixture was heat inactivated at 70ºC for 10 minutes before chilling on ice.

Reverse transcription PCR (RT-PCR) amplification

RT-PCR assays were carried out using primer pairs targeting the various genes (26 *kDa* and *ureA*), shown in Table 3.1.

3.11.4 Sample preparation for 2D gel electrophoresis

Two-dimensional (2D) gel electrophoresis is a powerful and widely used method for proteome analysis (Jung *et al.*, 2000; Bumann *et al.,* 2001; McAtee *et al.,* 2001). In this study, this technique was used to obtain and compare the protein profiles of the progressing aged cultures (morphological conversion from spiral form to the coccoid form).

Sample preparation

Culture cells at various time points were harvested and lysed using the lysis buffer (~300–400 µl) (Appendix A7.1). This was left on ice for 30 minutes. Endonuclease (Sigma) was added to the lysate and incubated on ice for 10 minutes. After which, the lysate was spun at 12,000 x g for 5 minutes at 4ºC. The supernatant was aliquoted and stored at -20ºC. The protein concentration for each extract was measured using the Bio-Rad Protein assay and bovine serum albumin (BSA) was used as the standard.

3.11.5 IEF and 2D PAGE

The IPG strip (Amersham Biosciences) was rehydrated prior to IEF (Isoelectric focusing). Protein samples were analyzed on 18 cm IPG strip with pH interval of 3-10 with a non-linear gradient (pH 3-10 NL) (Amersham Biosciences). The protein sample was diluted with rehydration buffer (Appendix A7.2) to a final total volume of 350 µl with 60 µg of protein. The rehydration buffer together with protein sample was loaded onto the strip holder. The strip was placed with the gel side down. To minimise evaporation and urea crystallization, IPG cover fluid was applied into the strip holder. Rehydration proceeded for 12 hours at a low voltage of 30 V (active rehydration) to facilitate the entry of the proteins. IEF was conducted using the Ettan IPGhor system with setting: 200 V for 200 Vhr, 500 V for 500 Vhr, 1000 V for 1000 Vhr, 8000 V for 62,000 Vhr for the 18cm strip used.

Prior to the run in second step, the strip was equilibrated with SDS buffer to transform the focused proteins into SDS-protein complexes which carries negative charges only. Equilibration was performed twice on a shaker for 15 minutes each. The first step was in 10 ml/strip of SDS equilibration buffer (Appendix A7.3) with 1% dithiotheitol (DTT). After the first 15 minutes, the strip was equilibrated with the equilibration buffer supplemented with 2.5% iodoacetamide (IAA) for another 15 minutes. Strip was then embedded on top of the 12 % SDS-PAGE gel using 0.5% (w/v) hot agarose embedding solution coloured with a trace amount of bromophenol blue. The protein marker was spotted onto the electrode pad and placed next to the strip. The vertical gel was run with low current (8 mA/gel) for the first 30 minutes to reduce the endoosmosis effects,

followed by increasing the speed of run to 25 mA/gel for ~5 hours or until the blue dye reached the bottom of the gel.

3.11.6 Visualisation of gels

After electrophoresis, the gel was removed from the gel cassette in preparation for staining. Two detection methods (coomassie blue and silver stains) were employed and procedures are as described in Section 3.3.3. As coomassie staining is a less sensitive stain in comparison to silver staining, the protein load was increased (> 500 µg/gel) when using coomassie stain. The stained gel was scanned using the Densitometer 710 (Bio-Rad) and protein profiles were compared and evaluated using the Bio-Rad image evaluation software, PD Quest.

3.11.7 Difference gel electrophoresis (DIGE)

DIGE is a method which pre-labelling the protein samples with fluorescent dyes such as Cy2, Cy3 and Cy5 prior to 2D electrophoresis for differential expression analysis (Yan *et al.*, 2002; Zhou *et al.*, 2002) (Figure 3.2). For this study, 3 and 30 day old RH 54 cultures were compared and analysed. The procedure was carried out according to the Ettan DIGE User manual (Amersham Biosciences).

Preparation of cell lysate compatible with CyDye DIGE fluor labelling

Bacterial cells were harvested by centrifugation at 12 000 x g for 4 minutes at 4ºC. The pellet was resuspended in 1 ml of cell wash buffer (Appendix A8.1) in a microfuge tubes. Cells were washed and centrifuged at 12 000 x g for 4 minutes at 4ºC. The pellet was again resuspended in wash buffer and the washing steps were repeated at least 3

times. The washed cell pellet was lysed with the cell lysis buffer (Appendix A8.2) and left on ice for 10 minutes. After which, the cells were subjected to 15s bursts from an ultrasonic probe (Soniprep 150 sonicator). Following 5 cycles of bursts interspersed with 1 minute cooling periods in ice at the end of each cycle. The crude extract was centrifuged at 12 000 x g for 10 minutes at 4ºC to remove any unbroken cells and debris. Cell supernatant was then retained and the pH of the lysate was checked by spotting 3 µl onto a pH indicator strip. The pH of the lysate would be adjusted to pH 8.0 with diluted sodium hydroxide (50 mM). Protein was quantified using Bio-Rad Protein Assay.

Preparation of CyDye DIGE fluors for protein labelling

The Cy2 and Cy3 were reconstituted in fresh 99.8% anhydrous dimethylformamide (DMF). The CyDye DIGE fluors were taken from the freezer and left to warm at room temperature for 5 minutes. A total of 25 µl DMF to 25 nmol of fluor. The mixture was mixed vigorously for 30 seconds and subsequently centrifuged for 30 seconds at 12, 000 x g using a microfuge. This was the stock fluor solution of 1nmol/µl. The fluors were stored in the dark and at -20ºC. This stock was diluted to working solution before use with the samples.

A 50 µg of protein was labelled with 400 pmol of fluor. The fluor and protein sample were thoroughly mixed by vortexing and were left on ice for 30 minutes in the dark. The reaction was stopped by the addition of 1 µl of 10 mM lysine. This solution was left on ice in the dark for 10 minutes. Labelling of the protein was completed and the labelled sample was stored at -70ºC in the dark for 3 months.

Rehydrating the gel strips with protein samples

Equal amount of 50 µg each of Cy3 labelled spiral protein and Cy5 labelled coccoid protein were mixed with equal volume of the 2X sample buffer (Appendix A8.3). The total volume of labelled protein was made up to the volume (350 µl) required for 18 cm Immobiline strip using the rehydration buffer. IEF and subsequent 2D PAGE were carried out as described in Section 3.11.4. The 2D gel images were captured using the Typhoon 9200 (Amersham Biosciences). The Cy3 gel images were collected at an excitation wavelength of 540 nm and an emission wavelength at 590 nm whereas the Cy5 labelled gels were scanned at an excitation wavelength of 620 nm and an emission wavelength of 680 nm. The resulted images were viewed using the Fluorsep software (Amersham Biosciences).

3.11.8 Spots excision and identification by Mass Spectrometry

Protein spots of interest from 2D gels stained with Cy-dyes, coomassie blue and silver stain were selected for identification by mass spectrometry. Comparison of protein expression profiles of the spiral and coccoid forms, protein spots of interest were excised from the gels manually with a clean spatula. Individual excised spots were placed in clean eppendorf tubes and kept at -80ºC prior to enzymatic digestion.

Silver stained proteins were destained with chemical reducers as described by Gharahdaghi *et al.* (1999) before trypsin digestion. Briefly, the silver stained gel spots were rinsed twice with water, followed by washing the spots in water for 10 minutes by vortexing. The reactive substances of the chemical reducers are potassium ferricyanide and sodium thiosulphate. These chemicals destained the gel spots by removing the silver.

Stock solutions of the chemical reducer and 16 mg/ml of sodium thiosulphate were freshly prepared. A 1:1 ratio of the two stock solutions were mixed and 50 µl of this mixture was added to the gel pieces. The gel pieces were destained by gentle vortexing for 30 minutes at room temperature. The brownish colouration of the gels disappeared and the gel pieces were rinsed twice with water to stop the reaction. A quick spin was carried out after each wash. After rinsing with water, 100 µl of 100 mM ammonium bicarbonate was added to cover the gel pieces for 20 minutes with gentle agitation. Subsequently, the gels were cut into smaller pieces and washed with 50-100 µl of 50 mM ammonium bicarbonate in 50% acetonitrile at room temperature for 15 minutes. After incubation, the liquid was discarded and the gel pieces were submerged with 3-4 gel volume of acetonitrile for another 15 minutes. The tube containing the opaque white gels was centrifuged and supernatant discarded. The gels were dried in a speed vac concentrator (Heto Maxi dry lyo system).

The dried gels were sent to Mass Spectrometry service centre where enzymatic digestion using the trypsin enzyme was carried out. Peptide mass maps of tryptic digest peptides were generated using MALDI-TOF mass spectrometry and subsequent database searches (MS-FIT & Mascot) were performed for identification of the proteins.

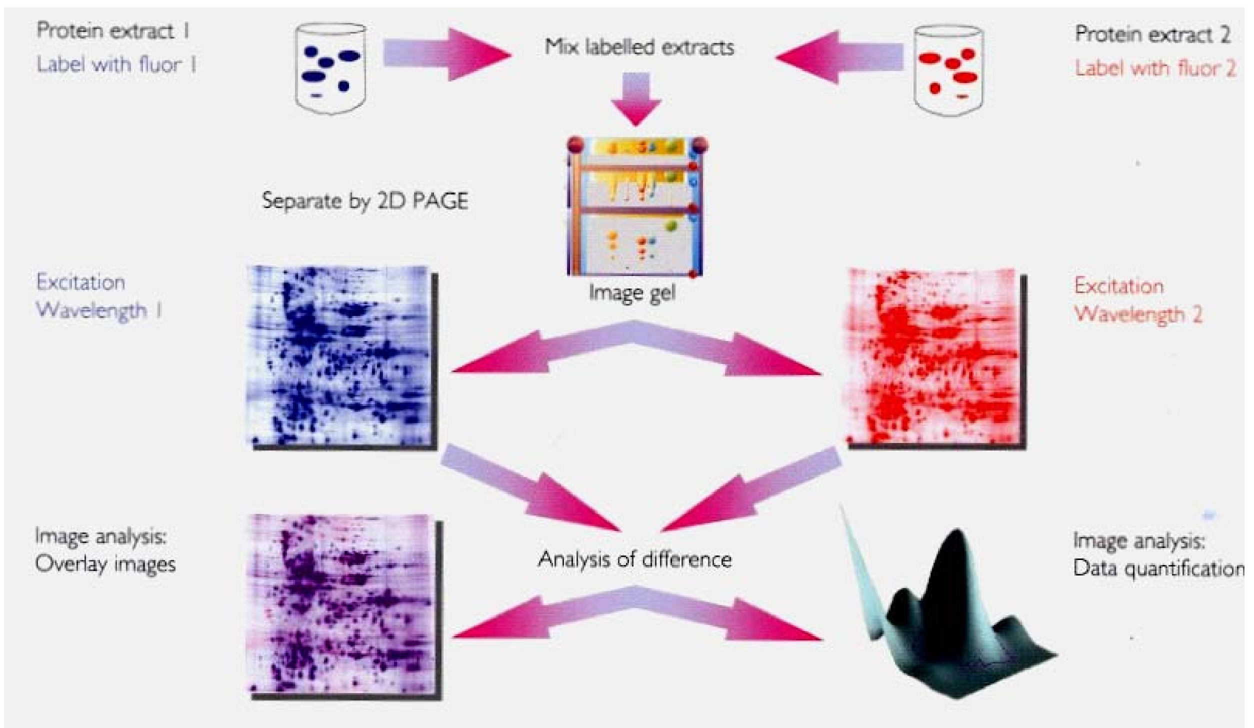


FIG 3.2 Flow chart of DIGE analysis of two protein samples. (Adapted from Amersham Biosciences)

RESULTS

4.1 BACTERIAL CULTURE

Figure 4.1 shows the growth of *H. pylori* on moist chocolate blood agar plate after incubation for 72 hours at 37 ºC in microaerophilic environment. *H. pylori* colonies were small (0.5-2.0 mm), translucent and water droplet-like.

4.1.1 Microscopy observation

Figure 4.2 shows the 2 differentiated morphological forms of *H. pylori* observed under phase contrast microscopy. In young culture (3-day old), *H. pylori* appeared as curved rods, while on prolonged incubation, coccoids were observed.

4.1.2 Gram staining and enzymatic tests

H. pylori is a Gram negative gastric bacterium (Figure 4.3). The bacterium is positive for urease, catalase and oxidase tests.

4.2 *H. pylori* IN FAMILIAL CLUSTER STUDY

4.2.1 Seroprevalence of *H. pylori*

In the familial cluster study, the distribution of sero-status of the 72 test subjects from 21 families was determined using our in-house ELISA assay. Among which, 50/72 (69.4%) were seropositive for *H. pylori* antibody IgG (Table 4.1). Seropositivity was more prevalent in the older family members of the same household. As shown in Figure 4.4, the prevalence increased with age from 50% among those with age from 1-15 years old to 100% seropositive in subjects aged 61-73 years old. It was observed that of the 19

FIG 4.1 The typical appearance of *H. pylori* colonies on a chocolate agar plate. Colonies are small (0.5-2 mm in diameter), translucent and water droplet like.

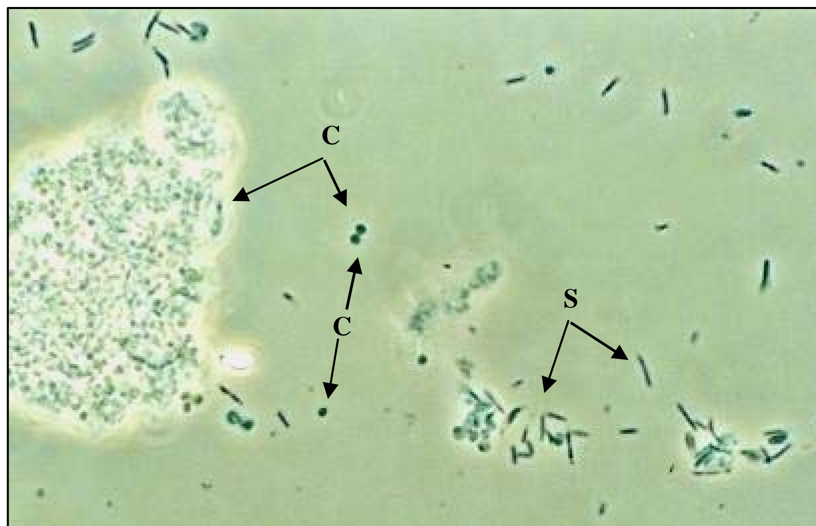
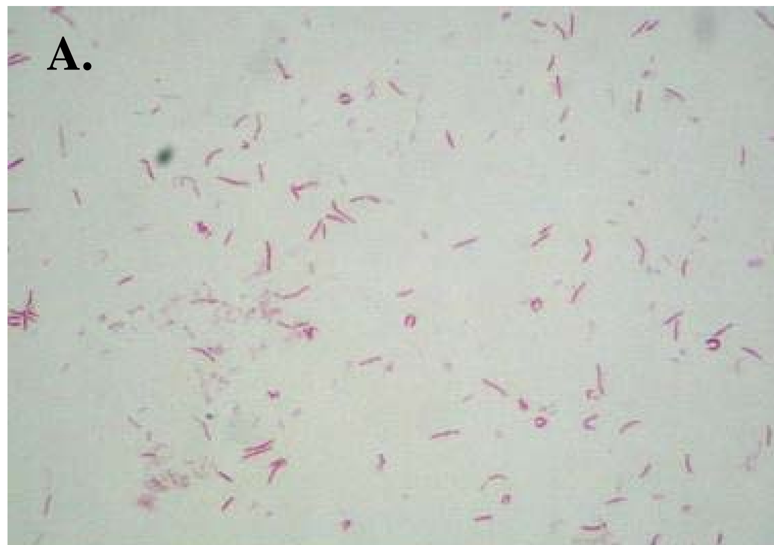


FIG 4.2 Dimorphic features of *H. pylori*. (1000 X) Phase contrast micrograph of a 4 day old culture, showing the different morphologies of *H. pylori*. (S: spiral, C: coccoid)



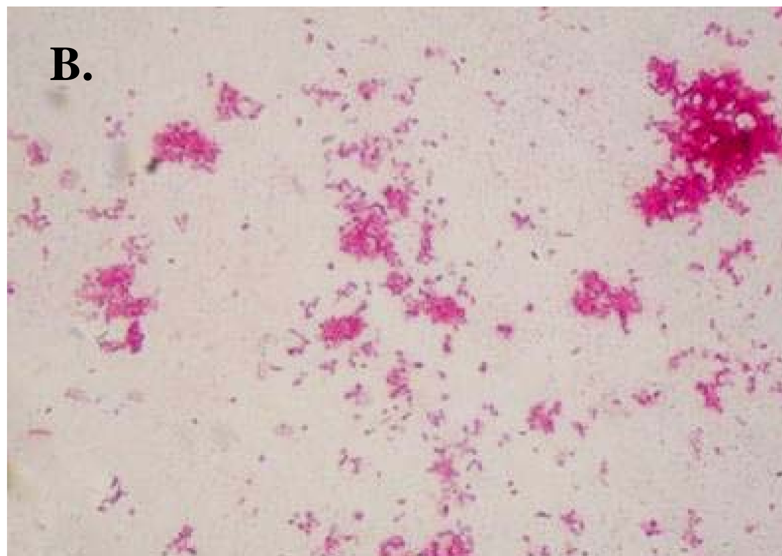


FIG 4.3 Gram staining of *H. pylori* (1000 x).
A: Spiral form (3 day old culture); B: Coccoid Form (30-day old culture).

families with at least one seropositive member, one or more of the younger members of the same family were also found to be seropositive. On the contrary, Family 6 which was made up of only 3 family members, the ELISA results showed that the child was seronegative even though both parents were tested seropositive. However, both husband and wife did not share similar antigenic profile. Besides this family, the young grandchildren in Family 10 were also tested seronegative when their grandparents were tested positive for *H. pylori* antibody. The remaining 2 families, Family 18 and 21 had no seropositive individuals (Table 4.1), thus these families were chosen as the negative controls for subsequent western blotting analysis.

4.2.2 Western blotting profiles

The antibody profiles showed a diversified pattern with protein masses ranging from ~8 to 130 kDa (Figure 4.5). It was observed that the older family members exhibited more reactive bands as compared to those younger members residing in the same household. Analysis of the immunobot profiles showed the presence of a high molecular mass protein of 120-130 kDa in 49/50 of the seropositive samples (Figure 4.5). Comparisons of the seropositive and seronegative immunoblots revealed the presence of a common group of proteins of medium molecular masses (~40-60 kDa).

4.2.3 Family cluster statistical analysis

Computer assisted analysis of the protein bands was performed using the Quantity One software program (Bio-Rad) and the agreement between antibody profiles of seropositive samples in the same household was assessed statistically using Cohen's

Table 4.1 Family cluster statistical analysis

Family	No. of family members	Seropositive members	* Comparisons with agreement (%)	Family relationship (for Cohen's kappa statistical analysis)
1	6	2	0/1 (0%)	-
2	5	4	1/6 (17%)	Mother & son
3	3	3	3/3 (100%)	Father & sons; siblings
4	2	2	1/1 (100%)	Siblings
5	3	2	1/1 (100%)	Mother & son
6	3	2	0/1 (0%)	-
7	4	4	6/6 (100%)	Grandmother & grandsons; Mother & daughter; mother & sons; siblings
8	3	2	0/1 (0%)	-
9	5	4	3/6 (50%)	Father & son; husband & wife; siblings
10	4	2	1/1 (100%)	Grandparents (refer to text)
11	4	2	1/1 (100%)	Mother & daughter
12	3	1	-	-
13	2	2	0/1 (0%)	-
14	3	3	0/3 (0%)	-
15	4	3	1/3 (33%)	Husband & wife
16	5	5	3/10 (30%)	Father & daughter; Father & son; grandfather & granddaughter
17	3	3	3/3 (100%)	Mother & sons; siblings
18	4	0	-	-
19	2	2	1/1 (100%)	Father & son
20	2	2	1/1 (100%)	Mother & son
21	2	0	-	-
Total	**72**	**50**	50 blots comparison	

$$* \text{ \% Similarity} = \frac{\text{No. of comparison with } \geq 40\% \text{ similarity}}{\text{No. of comparison between family members}} \times 100$$

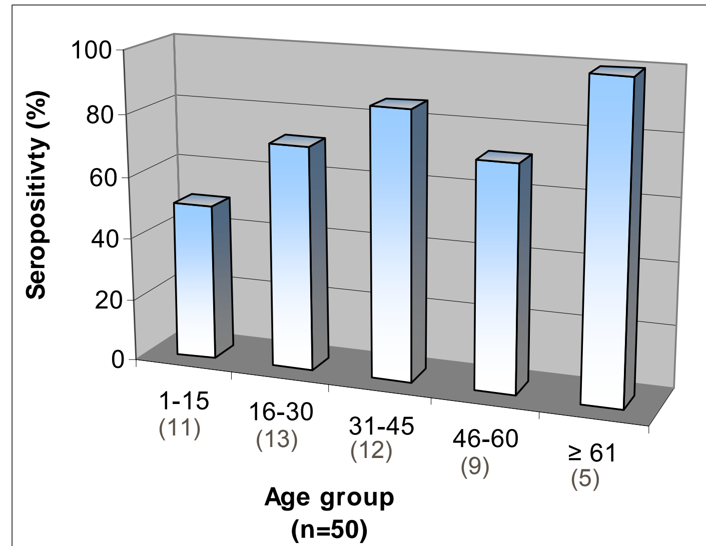


FIG 4.4 Seropositivity of *H. pylori* in different age groups.
Prevalence increased with age from 50% in those ages 1-15 to 100% in those age 61-73. Number in parenthesis denotes the seropositive samples in the respective age group.

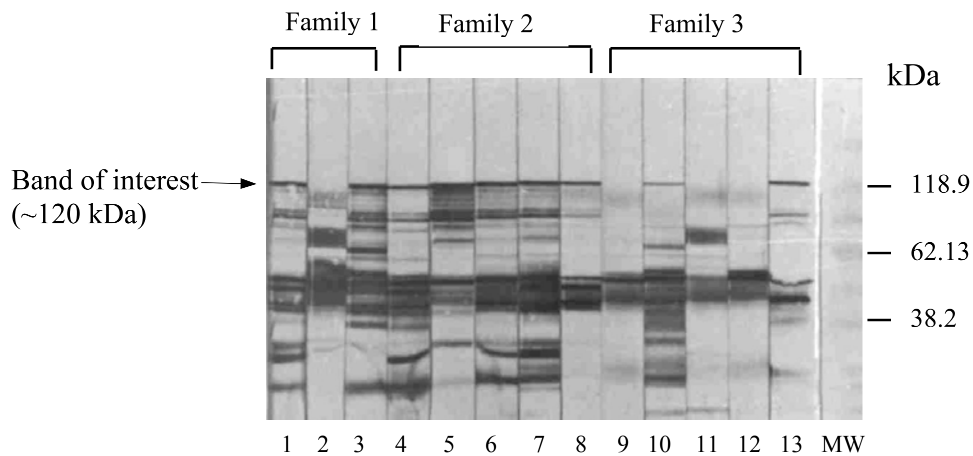


FIG 4.5 Immunoblot pattern obtained with sera from household members.
Seropositive samples: Lanes 1, 3, 4-8, 10 and 13; Seronegative samples: Lanes 2, 9, 11, 12. Arrow shows band of ~120 kDa present in seropositive samples. Lane 1, 2, 5-7, 9, 11, 12: younger members in individual household.

kappa analysis. The protein bands from the immunoblot were calibrated and comparisons of the blots between household members residing in the same family are illustrated in Table 4.1.

A total of 50 intra-familial blot comparisons were carried out for 18 families. No comparison could be made for the remaining 3 families. This is because no seropositive member was detected in members of 2 families (Families 18 and 21) while there was only one seropositive individual in Family 12. The percentage similarity as calculated according to Cohen's kappa analysis is shown in Table 4.1. Family 17 is used to illustrate the analysis. Of the 3 family members in this family who took part in the survey, all were tested seropositive (Mother and two sons). Meanwhile, a total of 3 comparisons were carried out; comparisons between Mother & son 1, Mother & son 2 and between siblings. From these 3 sets of comparison, it was noted all comparisons showed degree of similarity ≥ 40% (3/3). The relationships of those blots that demonstrated similarity were that of mother & sons and siblings as stated under the Family relationship column in Table 4.1.

Results from the present study showed that 5 families exhibited no similarity (0%) in the blot profiles compared. These are families 1, 6, 8, 13 and 14. The rest of the 13 families demonstrated a certain degree (17-100%) of agreement in protein patterns among individual household members.

Comparison of the 26 blots demonstrating similarity in blot profiles. Of these, 15 showed similarities in blot patterns between parents and children, 5 between siblings, 3 between grandparents and grandchildren and 3 between spouses.

It was noted that seronegative samples from Family 18 also showed immunoreactive bands (47-64 kDa). Therefore, comparisons of the positive samples

within each family after deducting those non-specific bands that were present in seronegative family 18 were also performed. It was then found that the results did not affect the overall findings.

4.3 *H. pylori* IN CHILDREN WITH EPIGASTRIC PAIN

4.3.1 Paediatrics cut-off point for ELISA

As there is no existing ELISA test kit specific for paediatrics, it is pertinent to have our in-house ELISA tested with a cut off value. The cut-off value for ELISA was based on a pre-evaluated comparative study of 114 local paediatric patients. Of these patients, 15 were culture and/or histology positive whilst the remaining 99 were negative for *H. pylori* infection by histology. Figure 4.6 illustrates the distribution of the IgG titre (OD values) for these two groups of children. The sensitivity and specificity of the serology assessments using various cut-off values are shown in Figure 4.7.

One of the ways to express the relationship between the sensitivity and specificity of the diagnostic test is to construct a Receiver-operator characteristic (ROC) curve (Kist *et al*, 1999; Kato *et al* 2003). Figure 4.7 depicts the ROC curve where the plot is based on sensitivity against 1-specificity. From this curve, the cut-off value of Mean+2sd (standard deviation) was deemed the most appropriate. With this cut-off value, the sensitivity and specificity were 100% and 97%, respectively.

Our ELISA data processing is based on a consistent set of positive control sample comprising the pooled sera of *H. pylori* infected patients with high IgG titre. Three sets of these sera were serially 2-fold diluted from 100X (P100) to 3200X (P3200). The ELISA for the control samples were carried out on separate plates on different

occasions so as to minimize any experimental errors. The chosen value of Mean+2sd (0.883) based on the ROC curve, fell within the absorbance of P400 and P800 (Table 4.2). The test results obtained were tabulated as such:

$OD_{sample} > OD_{p400}$: positive

$OD_{p400} > OD_{sample} > OD_{p800}$: grey zone

$OD_{sample} < OD_{p800}$: Negative

As the coccoid form is known to be viable but non-culturable (VBNC), the histology/culture results were based on observation/culturing of the spiral form. In view of this, the cut-off value for the spiral antigen was used in both ELISA test for spiral and coccoid antigens.

4.3.2 Seroprevalence of the two differentiated antigens

The serology results showed that 65 of 489 (13.3%) of the children with epigastric were found to have increased levels of IgG against the spiral form of *H. pylori*. In contrast, 273/489 (55.8%) were seropositive against the antigen prepared from the coccoid form (Figure 4.8). Of the 65 samples that were seropositive for the spiral-form antigen (spiral antigen), 36 were girls and 29 were boys. Interestingly, a proportional distribution of the seropositivity was also observed in 149 girls and 124 boys against the coccoid-form antigen (coccoid antigen). However, the distributions by gender for the groups seropositive for *H. pylori* spiral and coccoid forms did not differ significantly (P>0.05).

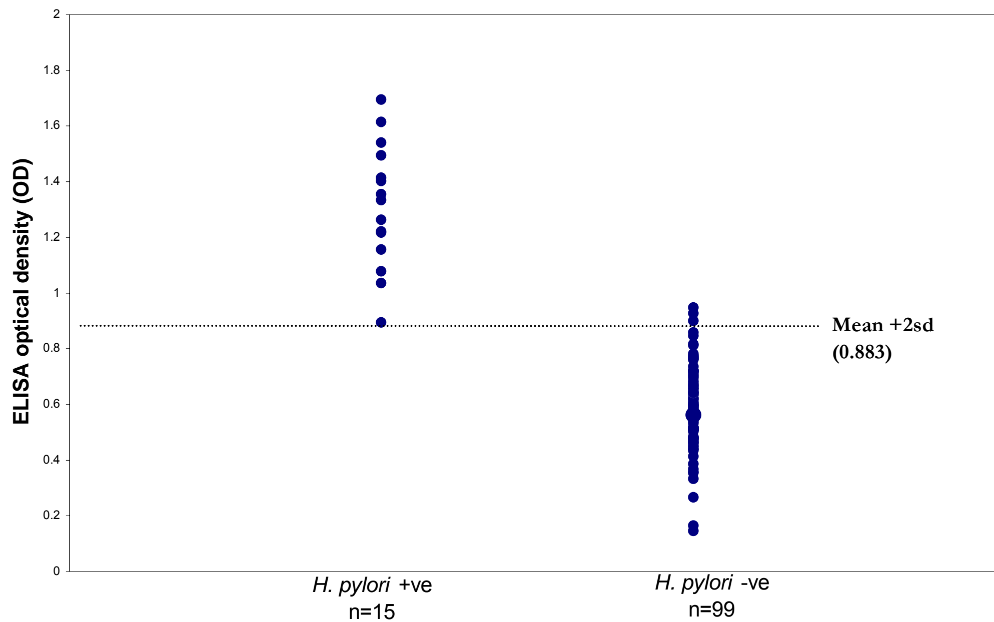


FIG 4.6 ELISA analysis of anti-*H. pylori* IgG in paediatric patients.

Distribution of IgG response (OD values) in *H. pylori* positive and negative children.

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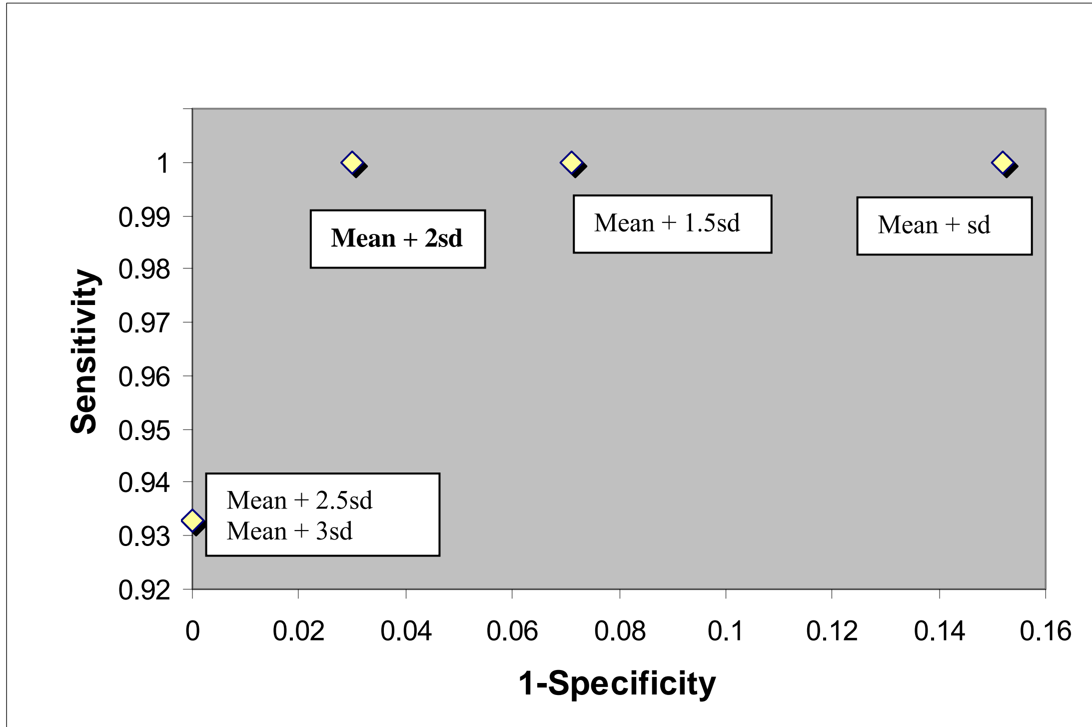
Table 4.2 Setting the cut-off limit for paediatric patients

	Absorbance readings for each plate (OD)			
Positive Control (Dilutions)	1	2	3	Ave
P100	1.277	1.455	1.547	1.426
P200	1.174	1.339	1.343	1.285
P400	0.929	1.013	1.021	0.987
P800	0.735	0.788	0.828	0.784
P1600	0.554	0.585	0.594	0.578
P3200	0.414	0.397	0.423	0.411

← **0.883**

Arrow shows Mean + 2SD = 0.883 (From FIG 4.6)



Cut-off	Sensitivity	Specificity
Mean + sd	1	0.848
Mean + 1.5sd	1	0.929
Mean +2sd	1	0.97
Mean +2.5sd	0.933	1
Mean +3sd	0.933	1

FIG 4.7 Receiver-operator characteristic (ROC) curve of calculated assay sensitivity and specificity at different cut-off levels.

According to this ROC curve, Mean+2sd provides the appropriate ELISA diagnostic value.

As shown in Table 4.3, among the 65 children who had raised levels of IgG antibodies against the spiral antigen, 58 were also found to have antibodies against the coccoid form of *H. pylori*. The remaining 7 samples seropositive for the spiral antigen were however found to have no significantly increased levels of IgG antibodies against the coccoid antigen. The serological results also showed that a total of 215 children tested seronegative for the spiral antigen were found to be seropositive for antibodies against the coccoid antigen. A total of 209 of 489 (42.7%) children in this study showed no antibodies against either form.

Of the 599 asymptomatic school children who represented the negative control in this study, 7% (42/599) had elevated levels of IgG antibodies against the spiral antigen and 26.5% (159/599) had antibodies against the coccoid antigen (Figure 4.8).

4.3.3 Use of coccoid antigen in serologic testing

To validate the use of the coccoid antigen as the antigen of choice for ELISA, the sensitivity and specificity of the assay were calculated using spiral antigen as the reference standard for comparison (Table 4.3). The choice of using the spiral antigen as the reference standard for comparison is because the spiral form is routinely used for the serodiagnosis of *H. pylori* infection. Furthermore, endoscopically confirmed *H. pylori* infection is also based on this actively dividing spiral form.

When spiral antigen was used as the standard for comparison, the calculated sensitivity was 89.2% (58 of 65 children) and specificity was 49.3% (209 of 424 children), with a negative predictive value of 96.8% (209 of 216 children) and a positive predictive value of 21.2% (58 of 273 children).

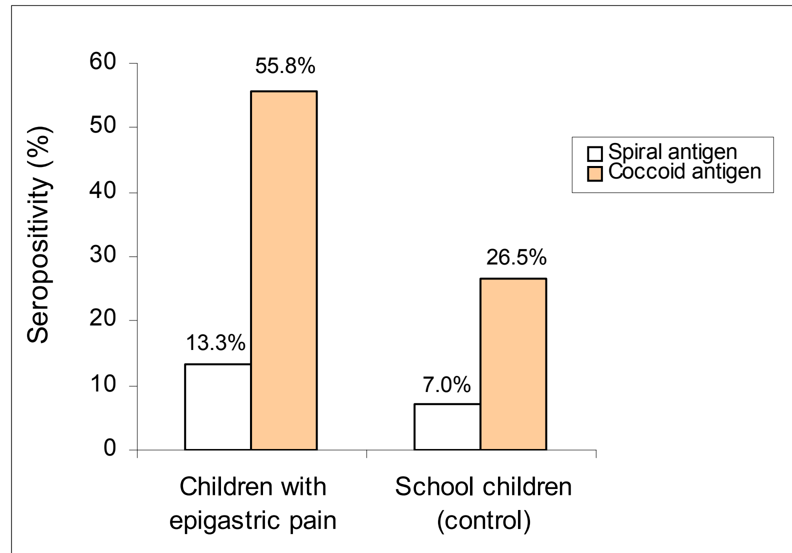


FIG 4.8 Comparison of seropositivity between school children and children with epigastric pain.
A total of 489 children with epigastric pain and 599 school children formed the cohort of the study.

TABLE 4.3 Seroprevalence of the two differentiated antigens of *H. pylori*

	Coccoid-form antigen	
Spiral-form antigen	Seropositive	Seronegative
Seropositive	58	7
Seronegative	215	209

* Following calculations were based on using the spiral-form antigen as standard for comparison.

Sensitivity: 58/65 = 89.2%

Specificity: 209/424 = 49.3%

Positive predictive value: 58/273 = 21.2%

Negative predictive value: 209/216 = 96.8%

4.3.4 Correlation of histology with seropositivity for coccoid antigen

Of 273 children with positive ELISA for the coccoid antigen, 120 underwent endoscopy. Interestingly, the histology findings showed that majority of these children (85/120) had evidence of inflammation (mild chronic gastritis). In addition, the presence of coccoid cells was detected in one patient. The remaining 35 patients demonstrated no significant histopathology their gastric antral mucosa.

4.3.5 Seroprevalence of paediatric patients of different ethnic origins

The children who participated in this study were of various ethnic backgrounds: Chinese, Malays, Indians and other ethnic groups. There were 360 Chinese, 43 Malays, 46 Indians and 40 of other races. The prevalence of seropositivity among the different races and age groups is illustrated in Table 4.4. Although there was no statistically significant difference in prevalence among the 4 ethnic groups ($P>0.05$), children of the Indian race were observed to have the highest rate of seropositivity (21.7%) for antibodies against the spiral antigen. Malay children appeared to have a slightly higher prevalence (14%) as compared to the Chinese children (12.5%) and children of other races (10%). The seroprevalence of *H. pylori* in various age groups is shown in Table 4.4. The study shows a gradual increase in the rate of acquisition of *H. pylori* infection with age. Children aged 5 years old and younger had a prevalence of seropositivity of 7.1% with prevalence increasing to 21.4% for those aged 11 years and older. The serology results for antibodies against the coccoid form (Table 4.5) showed that 54.2%, 60.5%, 60.9% and 60% Chinese, Malays, Indians and children of other races were seropositive, respectively. It was noted that 188/358 (52.5%) children of aged 10 and younger, were seropositive for

the coccoid-form antigen, whereas 85/131 (64.9%) of children aged 10 years and older were seropositive for coccoid antigen (Table 4.5).

4.3.6 SDS PAGE of spiral and coccoid antigens

Differential protein profiles of the acid glycine extracted (AGE) spiral (3 day old culture) and coccoid (150 days old culture) proteins are represented in Figure 4.9. Proteins were separated by 1-DE using 12% SDS-PAGE gel, subsequently stained by Coomassie G-250.

Comparison of the profiles showed that surface proteins of the two morphological forms were highly conserved. Nevertheless, a reduction of protein bands was observed in the coccoid culture. Both forms expressed major proteins with masses of 88, 69, 43, 24, 20, 17 and 16 kDa (Figure 4.9). The amount of proteins of 43, 24 and 16 kDa were significantly reduced in the coccoid cells. Two proteins of masses 33 and 13 kDa were expressed predominantly in the spiral cells. A protein of low molecular weight (MW), 20 kDa was up-regulated in the aged culture.

4.3.7 Immunoblot analysis of humoral immune response in children with epigastric pain

Western blots were used to detect *H. pylori* specific immunoreactive bands in 5 culture/histology positive and 4 culture negative samples. The positive samples were seropositive for spiral and coccoid antigens while the culture negative samples were seronegative for both antigens by ELISA.

As shown in Figure 4.10, variability in number and intensity of bands were observed in individual sample against the two differentiated antigens. In general, the total

number of bands detected was higher for *H. pylori* culture positive than the culture negative samples. The immunoreactive bands for the positive samples, ranged from 15 to 24 for the spiral antigen and 10 to 16 for the coccoid antigen. In comparison, the number of immunoreactive bands for the culture negative samples ranged from 7 to 11 bands for spiral antigen and 6 to 9 bands for the coccoid antigen. The cross reactive proteins detected in both the *H. pylori* positive and negative samples were mainly the medium molecular weight proteins of 45-67 kDa. When the immunoblot profiles were compared, several common *H. pylori* specific immunoreactive proteins were detected (Figure 4.10 1b): A (144 kDa); B (102 kDa); C (94 kDa), D (81 kDa), E (72 kDa), F (42 kDa); G (36 kDa) and H (32 kDa). Similarly, a number of low MW bands were also detected in the *H. pylori* positive samples (13-17 kDa). Figure 4.10 2b, reveals that the 2 morphological forms shared common immunoreactive bands of 144 kDa (Band A), 81 kDa (Band D) and 42 kDa (Band F) though the intensity of these bands were much reduced. Interestingly, a coccoid specific band of ~ 298 kDa (Figure 4.10 2b) was observed.

TABLE 4.4 Seropositivity to *H. pylori* spiral form antigen in children with epigastric pain

Age range (yrs)	Chinese (n=360)	Malays (n=43)	Indians (n=46)	Others* (n=40)	Total (n=489)
0-5	5/78 (6.4%)	1/6 (16.7%)	1/7 (14.3%)	0/7 (0%)	7/98 (7.1%)
6-10	19/188 (10.1%)	2/22 (9.1%)	5/26 (19.2%)	4/24 (16.7%)	30/260 (11.5%)
11-15	21/94 (22.3%)	3/15 (20%)	4/13 (30.7%)	0/9 (0%)	28/131 (21.4%)
Total	**45/360 (12.5%)**	**6/43 (14.0%)**	**10/46 (21.7%)**	**4/40 (10.0%)**	**65/489 (13.3%)**

* Others (other ethnic groups)

TABLE 4.5 Seropositivity to *H. pylori* coccoid form antigen in children with epigastric pain

Age range (yrs)	Chinese (n=360)	Malays (n=43)	Indians (n=46)	Others* (n=40)	Total (n=489)
0-5	43/78 (55.1%)	3/6 (50%)	4/7 (57.1%)	4/7 (57.1%)	54/98 (55.1%)
6-10	91/188 (48.4%)	11/22 (50%)	17/26 (65.4%)	15/24 (62.5%)	134/260 (51.5%)
11-15	61/94 (64.9%)	12/15 (80%)	7/13 (53.8%)	5/9 (55.6%)	85/131 (64.9%)
Total	**195/360 (54.2%)**	**26/43 (60.5%)**	**28/46 (60.9%)**	**24/40 (60.0%)**	**273/489 (55.8%)**

* Others (other ethnic groups)

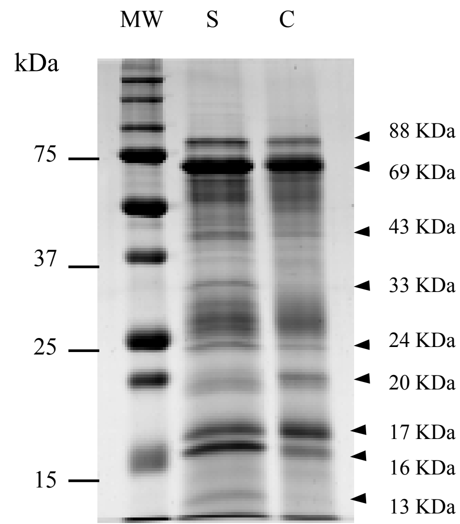


FIG 4.9 Protein profiles of the spiral (S) and coccoid (C) AGE antigens.
Proteins separated by 1-DE, followed by coomassie blue staining.
Arrows indicate the common/ unique proteins bands.

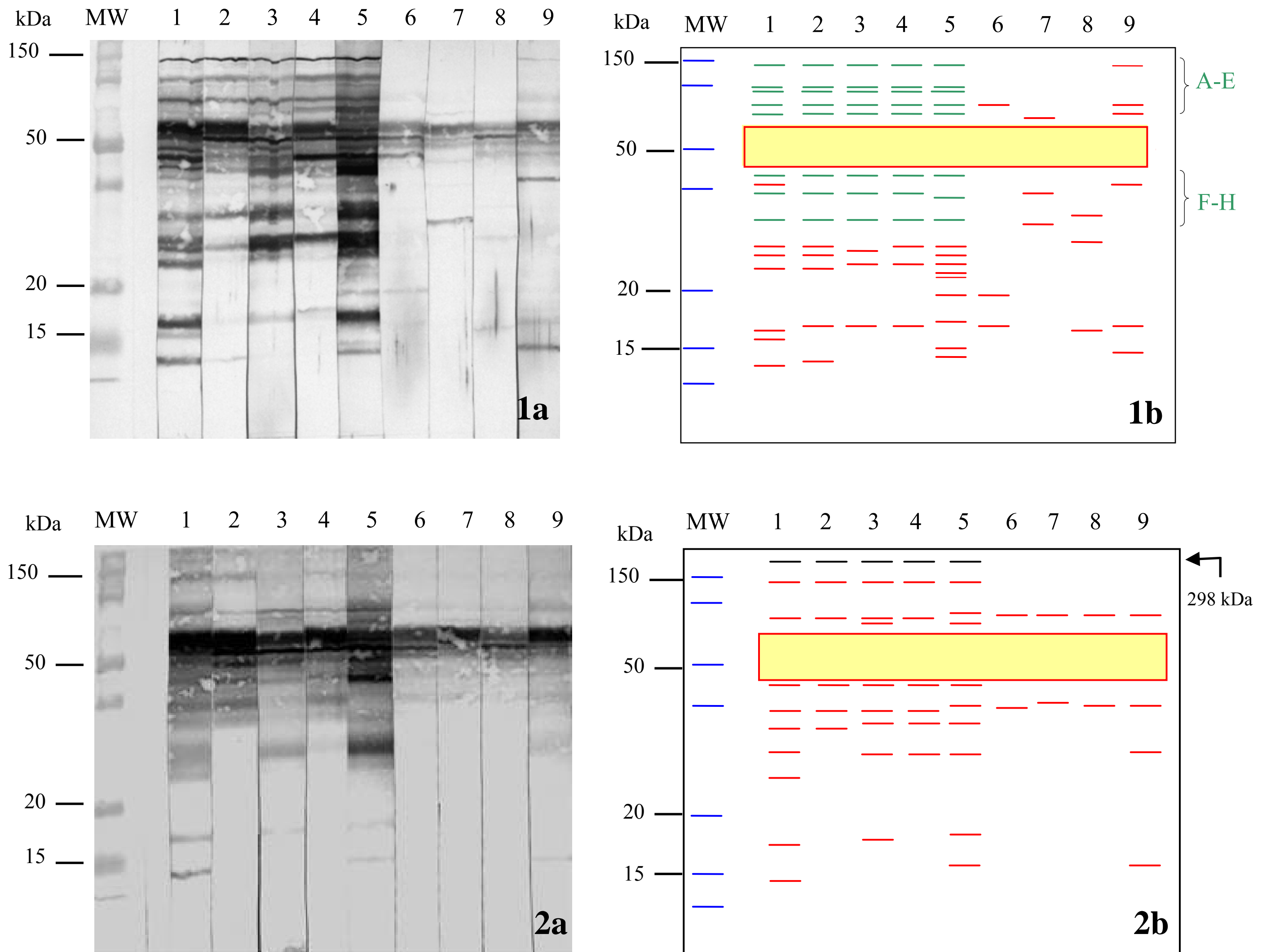


FIG 4.10 Western blot patterns of sera from 9 dyspeptic patients.

1a & 2a are immunoblot patterns against the spiral-form and coccoid-form antigens, respectively, while 1b & 2b are the diagramatic representations of the blots, respectively. Lanes 1-5 show the patterns blotted with sera of *H. pylori* culture positive patients. Lanes 6-9 show the patterns blotted with sera from patients who were *H. pylori* culture negative. Identification of several immunoreactive bands: A (144 kDa); B (102 kDa); C (94 kDa), D (81 kDa), E (72 kDa), F (42 kDa); G (36 kDa); H (32 kDa), highlighted in dark green. In 2b, bands highlighted in black (298 kDa) is seen present in positive samples against the coccoid antigen.

4.4 CLINICAL STRAINS FROM PAEDIATRIC PATIENTS

The 15 *H. pylori* isolates were shown to be spiral form (Figure 4.3A) from a 3 day old culture grown on the chocolate blood agar plates (Appendix A1.2 or A1.3) or embedded in the gastric antral biopsies (Figure 4.11). The organism was positive for urease, catalase and oxidase tests.

The clinical findings of the 15 isolates are depicted in Table 4.6.

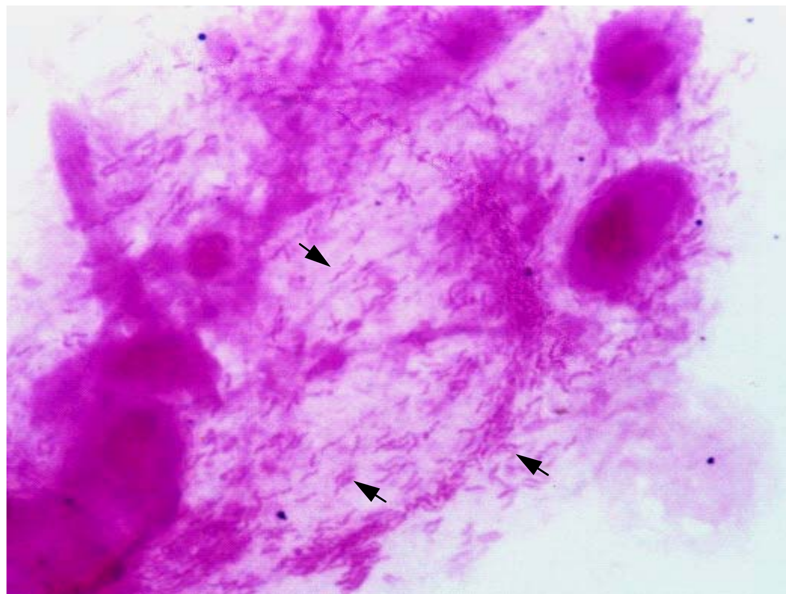


FIG 4.11 Gram stain of a *H. pylori* positive biopsy smear.
Large number of *H. pylori* in gastric biopsy stained in pink.
Arrows indicate the Gram negative spirals.

Table 4.6 Clinical findings of the 15 isolates

Strain No.	Biopsy code no.	Endoscopy diagnosis
1	299	Duodenal ulcer
2	310	Duodenal ulcer
3	393	Gastritis
4	504	Gastritis
5	522	Gastritis
6	578	Gastric ulcer
7	741	Non-ulcer dyspepsia
8*	1260	Duodenal ulcer
9*	1272	Healed duodenal ulcer
10	1276	Non-ulcer dyspepsia
11	1295	Esophagitis
12	1320	Gastritis
13	1351	Gastritis
14	1352	Gastritis
15	1416	Duodenitis

* isolated from same patient

4.4.1 RAPD PATTERNS

4.4.1.1 Genomic fingerprinting of clinical strains

RAPD profiles generated using the 10bp primer 773 was found to be useful in strains discrimination. Figure 4.12 illustrates the RAPD-DNA fingerprints obtained with the 15 clinical isolates from the symptomatic children. Each isolate presented a total of 4 to 7 bands of MW ranging from 0.4 kbp to 3.0 kbp. It was observed that each patient harboured a distinct *H. pylori* isolate even though commonly conserved bands were seen in some isolates. As shown in Figure 4.12, strains 8 and 9 displayed similar DNA profiles. Strain 9 was isolated from patient 8 during the revisit 2 months later.

4.4.1.2 Genomic fingerprinting of clones

In order to assess whether there is multiple-strain colonization in local population, 5 single colonies (single colony strains) were randomly isolated from each of the 5 different clinical cultures. In all, chromosomal DNA of 25 single colonies were extracted and typed. The 5 clones of each respective culture exhibited identical DNA fingerprinting patterns. Figure 4.13 illustrates the representative profiles from 2 of the 5 clinical strains. Similarity in DNA profiles between clones suggests that the patients harboured predominantly single *H. pylori* strain.

4.4.2 PREVALENCE OF VIRULENCE GENES

The presence or absence of the virulence genes in the 15 *H. pylori* isolates was analysed by PCR (Figure 4.14) with the specific primers (Table 3.1 & 3.2).

4.4.2.1 *H. pylori* genotypes in paediatric patients

The *cagA* and *cagE* genes were detected in all 15 isolates (Table 4.7). The *iceA* genotyping revealed that 12 of 15 isolates (80%) were positive for *iceA1* while *iceA2* was found in the remaining 3 strains. Interestingly, these 3 isolates were isolated from patients who had gastritis. The *babA2* gene was detected in 12/15 (80%) isolates.

The allelic variation of *vacA* gene was studied (Table 4.8). The *vacA* s1 allele was present in all strains tested. The s1c was observed to be the most common s alleles with 13 strains possessing the gene. Three strains were detected with s1a alleles. The s2 allele was observed only in 1 *H. pylori* isolate. The analysis revealed no s1b type in this study.

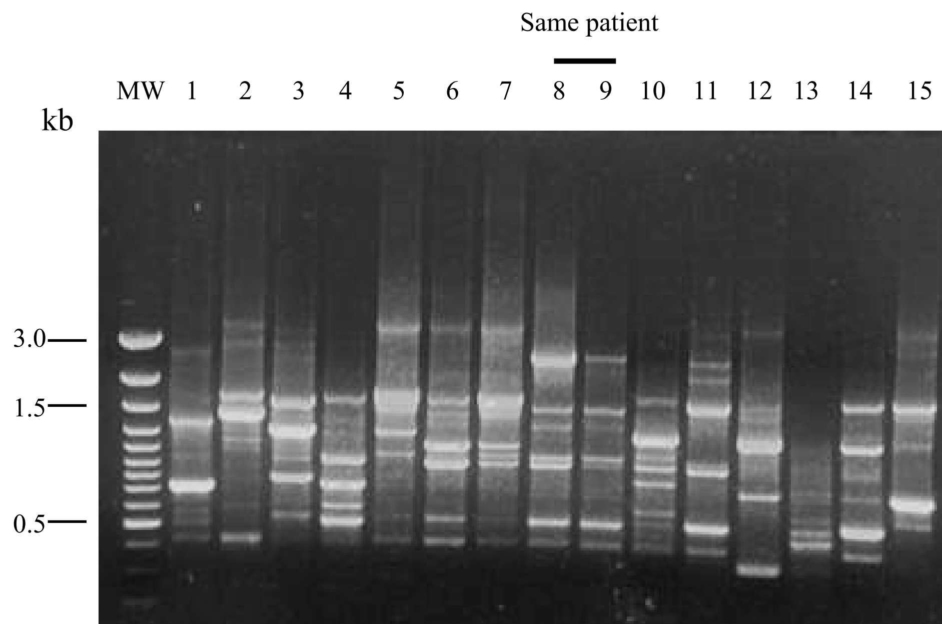


FIG 4.12 Arbitrarily primed PCR (RAPD) fingerprint patterns of *H. pylori* isolates.
The PCR profiles of 15 clinical isolates generated using primer 773. Unique fingerprints were observed for each isolate except isolates 8 and 9. Isolate 9 was isolated from patient 8 on a revisit. MW shows the DNA size marker (GeneRuler™ 100bp DNA Ladder Plus).

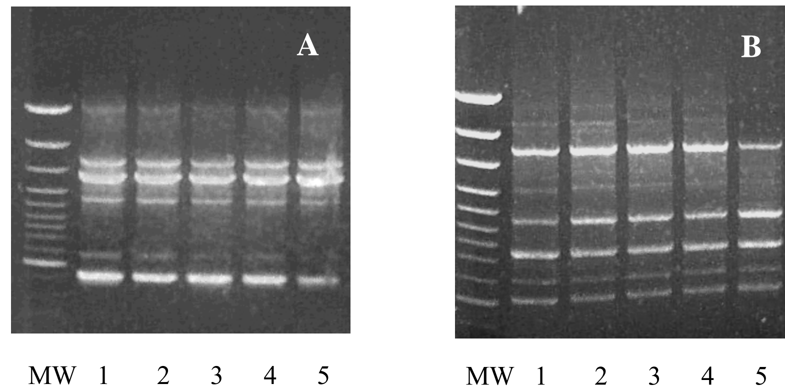


FIG 4.13 Representative RAPD patterns of various clones of 2 clinical isolates.
Clones of each isolate (A: Strain 2; B: Strain 4) displayed similar RAPD patterns.

Type m2 was detected in 7 strains while m1T was found in 4 strains. As for the remaining 4 isolates, 2 were found to be of m1 genotype, whilst the other 2 strains were assigned for m1Tm2 genotype (Table 4.8). Interestingly, 2 strains (Strains 10 and 12) were found to have mixture of alleles for the s regions (Table 4.8). Considering the combination of *vacA* s and m types, 6 mosaic combinations were found (Table 4.9) with genotype s1c/m2 being the most frequently observed in paediatric patients with a frequency of 37.5% (6/16).

Overall, the common genotype of strains isolated from local young patients is observed to be *cagA*+, *vacA* s1c/m2 *iceA1* and *babA2*+. The only s2/m2 strain was isolated from a patient with NUD and the genotype was detected to be *cagA*+, *vacA* s1c/s2/m2, *iceA2* and *babA2* -.

4.4.2.2 Virulence genotypes of clones from single colony

Twenty-five single colony samples from 5 clinical isolates were typed for various virulence genes. The PCR results showed that all single-colonies obtained from an isolate displayed similar genotype. Figure 4.15 depicts the virulence genotypes of the 5 clones from clinical strain 9. This strain was found to be positive for *cagA*, *vacA*, *iceA1* and *babA2* genes.

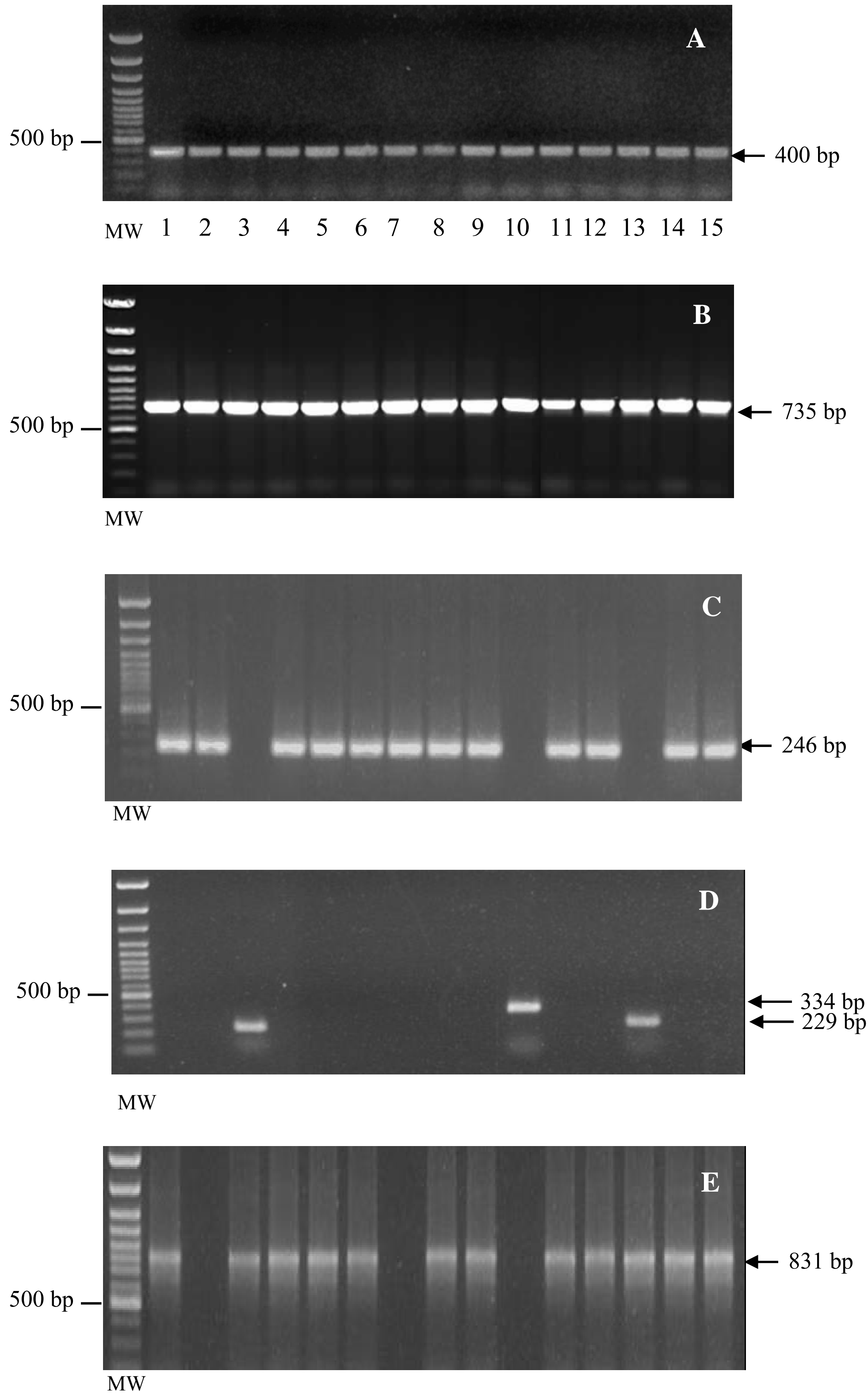


FIG 4.14 PCR products of various genes.
A 1% agarose gel A stained with ethidium bromide illustrating the PCR products for A: *cagA*; B: *cagE*; C: *iceA1*; D: *iceA2* and E: *babA2*. MW shows the DNA size marker (GeneRuler™ 100bp DNA Ladder Plus).

Table 4.7 Genotypes of the 15 clinical isolates

Strain No.	Genotype status				
	cagA	***cagE***	***iceA1***	***iceA2***	***babA2***
1	+	+	+	-	+
2	+	+	+	-	-
3	+	+	-	+	+
4	+	+	+	-	+
5	+	+	+	-	+
6	+	+	+	-	+
7	+	+	+	-	-
8	+	+	+	-	+
9	+	+	+	-	+
10	+	+	-	+	-
11	+	+	+	-	+
12	+	+	+	-	+
13	+	+	-	+	+
14	+	+	+	-	+
15	+	+	+	-	+

(+) refers to amplification of the respective gene regions by PCR

Table 4.8 ***H. pylori vacA*** **genotypes**

Strain No.	***vacA*** **alleles**							
	m1	**m1Tm2**	**m1T**	**m2**	**s1a**	**s1c**	**s1b**	**s2**
1	-	-	+	-	-	+	-	-
2	-	-	-	+	-	+	-	-
3	+	-	-	-	+	-	-	-
4	-	-	+	-	-	+	-	-
5	-	-	-	+	-	+	-	-
6	-	-	+	-	-	+	-	-
7	-	-	-	+	-	+	-	-
8	-	+	-	-	-	+	-	-
9	-	+	-	-	-	+	-	-
10	-	-	-	+	-	+	-	+
11	-	-	+	-	-	+	-	-
12	-	-	-	+	+	+	-	-
13	-	-	-	+	-	+	-	-
14	+	-	-	-	+	-	-	-
15	-	-	-	+	-	+	-	-

(+) refers to amplification of the respective m and s regions by PCR

Table 4.9 Relationships between m-region and signal sequence typing of *vacA* for 15 *H. pylori* isolates

Genotype(s)	No. of isolates
s1a/m1	2
s1a s1c/m2	1
s1c/m2	7
s1c/m1T	4
s1c s2/m2	1
s1c/m1tm2	2

4.4.3 ANTIMICROBIAL SUSCEPTIBILITY STUDY

In this study, the primary resistance of the 15 *H. pylori* isolates to amoxicillin, clarithromycin, metronidazole and tetracycline were tested using antibiotics sensitivity tests. These are frequently used antibiotics in combination with proton pump inhibitors or bismuth salts for the treatment of *H. pylori* infection. The antimicrobial susceptibility testing was first performed using the disk diffusion method (Figure 4.16A). The diameter of zone of growth inhibition was recorded and the cut-off for each antibiotic was referred to Table 3.3.

All 15 isolates were sensitive to amoxicillin and tetracycline by disk diffusion test. It was observed that 4/15 (26.7%) and 10/15 (66.7%) of the isolates were resistant to clarithromycin and metronidazole, respectively by disk diffusion test. The clarithromycin and metronidazole susceptibility testing was further evaluated using the E-test (Figure 4.16B). Two isolates (13.3%) was shown to be resistant to clarithromycin with an MIC of 8mg/L (cut-off for Clr® is at 2 mg/L). Resistance to metronidazole was observed in 8/15

(53.3%) strains using E-test. Of the 8 metronidazole resistant isolates, 1 exhibited MIC of 32 mg/L and remaining 7 had higher MICs (>256 mg/L) (cut-off for Mtr® is at 8 mg/L). A higher resistant rate was observed using the disk diffusion method as compared to using the E-test. Nonetheless, strains tested resistant by E-test were also noted to be resistant by disk diffusion method.

The antimicrobial susceptibility of the two strains (Strains 8 & 9) isolated from same individual on 2 different occasions showed that the strain isolated during the second visit exhibited combined resistance to clarithromycin (8 mg/L) and metronidazole (>256 mg/L). This is the only strain observed in the present study that demonstrated resistant to clarithromycin and metronidazole.

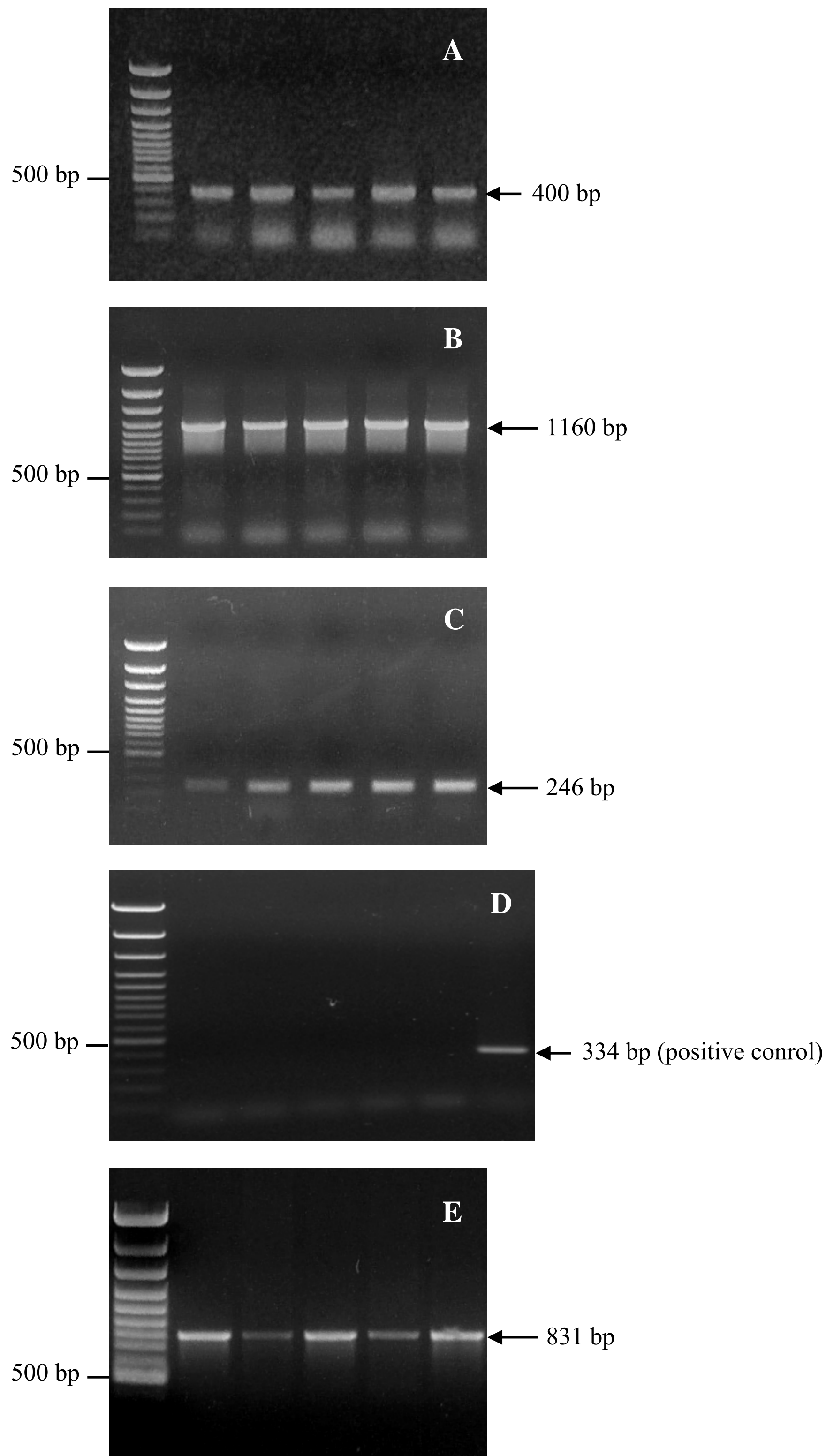


FIG. 4.15 PCR products of various genes of *H. pylori* strain 9

A 1% agarose gel showing the PCR products of A: *cagA*; B: *vacA*; C: *iceA1*; D: *iceA2* and E: *babA2*. Strain 9 is positive for *cagA, vacA, iceA1 and babA2*. MW shows the DNA size marker (GeneRuler^{TM} 100bp DNA Ladder Plus).

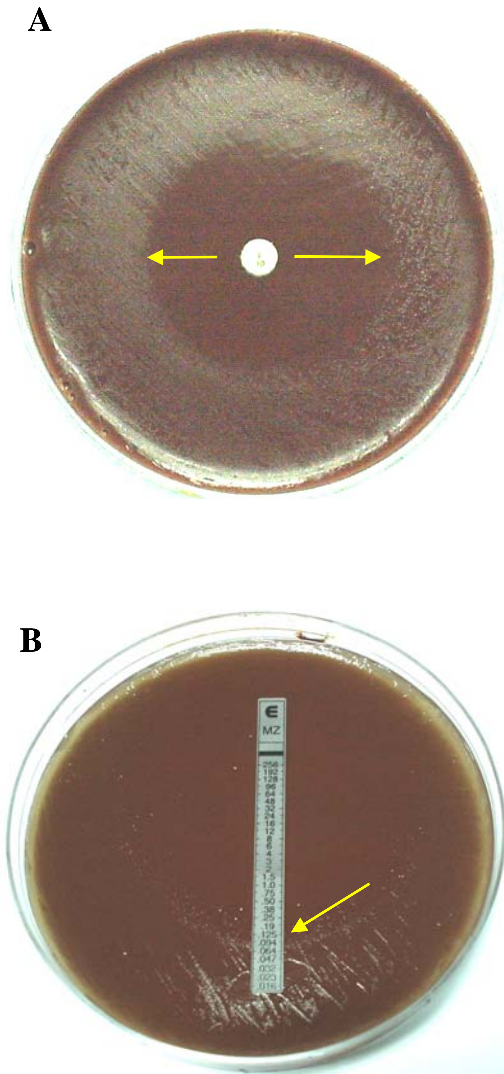


FIG 4.16 Antimicrobial susceptibility tests.
A: Disk diffusion method (arrow indicates the zone of inhibition)
B: E-Test (arrow indicates the point of inhibition of growth)

4.4.4 EXPRESSION OF LEWIS ANTIGENS

4.4.4.1 In primary *H. pylori* isolates

Le antigen typing on 15 *H. pylori* isolates using ELISA showed that all the isolates expressed Le^x while 13/15 (86.7%) isolates expressed Le^y. Only 1 isolate was found to express the Le^a and 3/15 (20%) were shown to carry the Le^b antigen. Present study showed that the type II carbohydrate antigens, Le^x and Le^y are more commonly expressed by the *H. pylori* isolates than the type I antigens, Le^a and Le^b. In addition, 14/15 isolates were observed to express ≥2 Le antigens. All isolates had both Le^x and Le^y except 1 strain which expressed Le^x and Le^a instead.

The *H. pylori* strains (8 & 9) isolated from the same patient during different visits demonstrated not only similar genotype but also expressed homogeneous Le antigens phenotype. Both isolates expressed Le^x, Le^y and Le^b.

4.4.4.2 In aged *H. pylori* cultures

Le antigens expression by *H. pylori* during morphological conversion was studied using the standard strain (NCTC 11637) and a local clinical isolate (RH 54). RH 54 was grown from a period of 3 to 184 days while NCTC 11637 was grown for a slightly longer period of 219 days.

The Le antigens expression was based on the OD values as a measure of the amount of antigens expressed by the individual strain. Values >0.2 OD were considered positive (Zheng *et al*, 2000). Tables 4.10 and 4.11 illustrate the mean OD values of the 2 *H. pylori* strains tested. Both strains were observed to express different levels of Le antigens (Mean OD for RH 54 > Mean OD of NCTC 11637). The results showed that

RH 54 expressed Le^x and Le^y while NCTC 11637 expressed only Le^y. Neither of the strains expressed Le^a. Expression of Le^b by RH 54 was observed in 5-30 days old cultures.

When Le antigens expression at various time points were compared, 2 OD peaks were observed, at 5th and 30th day of the growth profiles (Figure 4.17). The general trend of Le antigens expression increased from the 3rd to 5th day of growth. After which a decline in the Le antigens level continue until the 14th day of growth. Interestingly, an increase in Le antigen level renewed from 14th to the 30th day. Subsequently, as the culture aged further (184/219 days), the antigens expression dropped to a level comparable to that of the 3 day old culture. This interesting pattern of Le expression during the morphological conversion is more pronounced in RH 54 (Figure 4.17).

4.5 IMMUNOELECTRON MICROSCOPY AND FLOW CYTOMETRIC ANALYSIS OF THE LOCALIZATION OF *H. pylori* ANTIGENS DURING DIFFERENT MORPHOLOGICAL STAGES

Transmission Electron Microscopy (TEM) was used to examine the antigen-antibody interaction with immunogold labelled rabbit anti-*H. pylori* antibodies against both morphological forms of *H. pylori* .

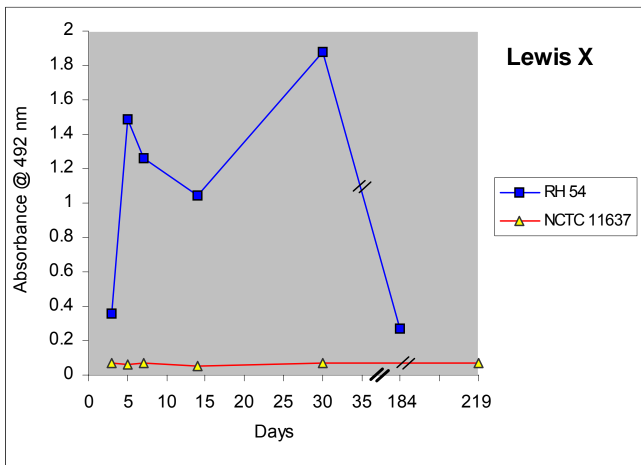
The electron micrographs (Figure 4.18A & B) show that immunogold particles of 10nm were seen to localize on the cell surface of 3 day old *H. pylori* regardless of the types of antibodies (anti-spiral antibodies & anti-coccoid antibodies) used. Similar observation was noted for the coccoid cells against the anti-spiral and anti-coccoid antibodies (Figure not shown).

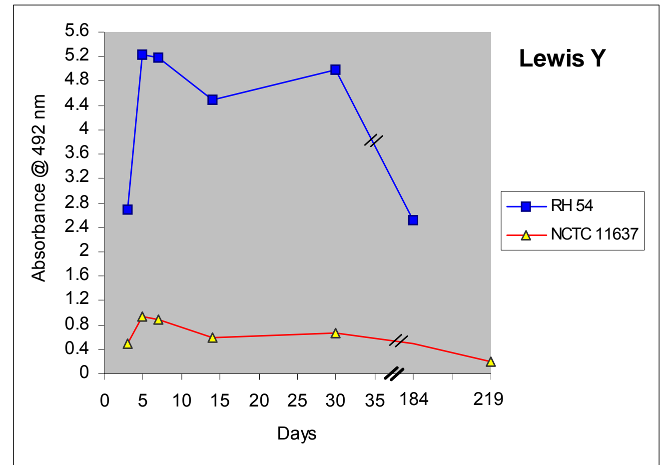
Table 4.10 Lewis antigens expression (Average OD value) in aging culture of *H. pylori* strain RH 54

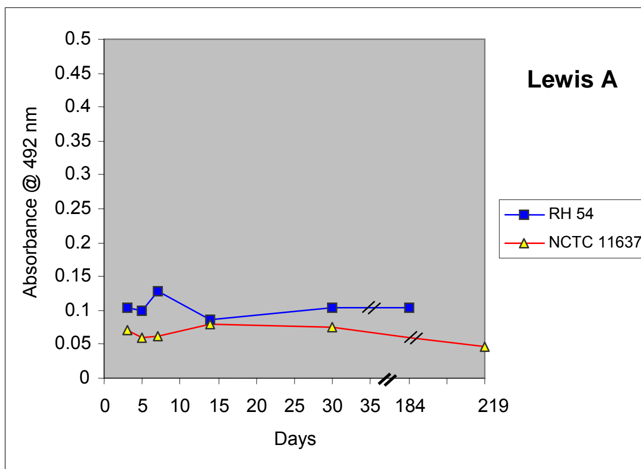
	Strain RH 54					
Le Ag / Days of culture	3	5	7	14	30	184
Le ^{x}	0.357	1.489	1.263	1.043	1.882	0.273
Le ^{y}	2.680	5.221	5.188	4.498	4.981	2.525
Le ^{a}	0.105	0.100	0.129	0.086	0.104	0.105
Le ^{b}	0.092	0.336	0.303	0.347	0.499	0.086

Table 4.11 Lewis antigens expression (Average OD value) in aging culture of *H. pylori* strain NCTC 11637

	Strain NCTC 11637					
Le Ag / Days of culture	3	5	7	14	30	219
Le ^{x}	0.069	0.060	0.065	0.053	0.074	0.074
Le ^{y}	0.487	0.932	0.883	0.601	0.664	0.207
Le ^{a}	0.070	0.060	0.063	0.079	0.076	0.046
Le ^{b}	0.076	0.202	0.163	0.123	0.149	0.045







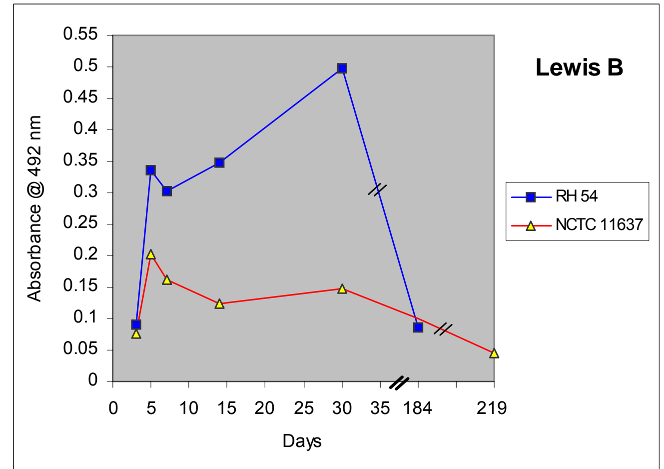


FIG 4.17 Graphical representations of the Le antigens expression during morphological conversion.

Two peaks, at 5th and 30th day of culture were observed. As the culture aged further, the Le antigens expression level dropped to that comparable to the 3-day old culture.

Based on the finding that the antigens were localized on the cell surface of *H. pylori*, flow cytometry was engaged to further examine the antigen-antibodies interaction. In Figure 4.19, the flow cytometry analysis illustrates the binding affinity of the purified antibodies to *H. pylori* spiral and coccoid cells. The anti-spiral IgG has a higher affinity for the 3-day old cells as compared to the 35 and 184-day cells, which comprised mainly the coccoid cells (Figure 4.19A & B). Similarly, the anti-coccoid IgG was incubated with both spiral (3-day old culture) and coccoid cells (35 and 184-day old culture). Interestingly, there is a shift of the coccoid cell profiles (35 to 184-day old culture) to the right as observed in graphs C and D. This observation illustrates a binding preference of the anti-coccoid IgG to the coccoid cells.

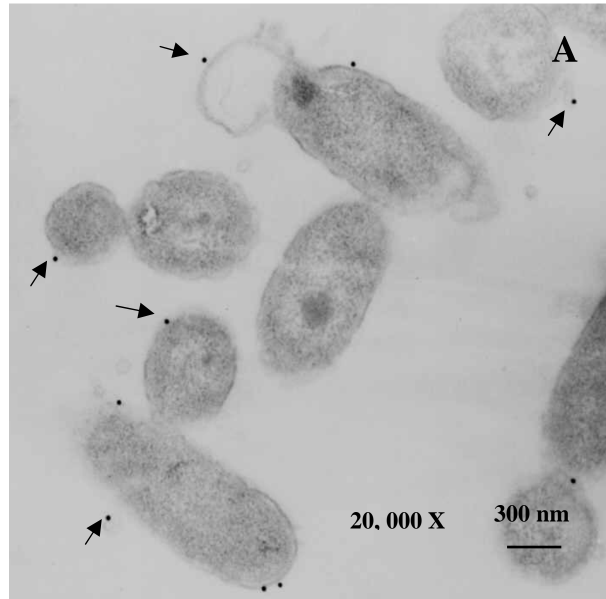
4.6 GENOME AND PROTEOME STUDIES

4.6.1 Genomic fingerprinting of aging RH 54 and NCTC 11637 cultures

As illustrated in Figure 4.12, RAPD fingerprints showed that the DNA patterns of the 2 strains were dissimilar. However, DNA profiles of the spiral forms were highly homologous to their respective coccoid forms (Figure 4.20). Both strains show slight variations in band density in aged culture of 184 days and 219 days. Conversely, majority of the bands were conserved in aging cultures which comprised the coccoid cells.

4.6.2 Presence and expression of various genes in aged cultures

The test strains at various time points were tested for the presence of the *26kDa*, *ureA, cagA and vacA* genes. Figure 4.21 illustrates the presence of PCR products of 294 bp and 411 bp in all cultures even up to a long period of 184/219 days which



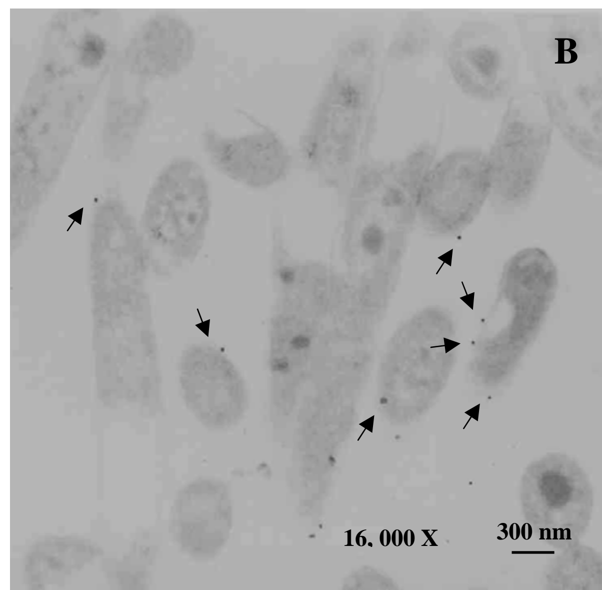


FIG 4.18 Electron micrographs showing binding of various antibodies to 3-day old *H. pylori* cells using the immunogold labeling technique.
A: anti-spiral IgG + 10nm immunogold particles. B: anti-coccoid IgG + 10nm immunogold particles. Arrows show the sites of localization of the labelled antibodies.

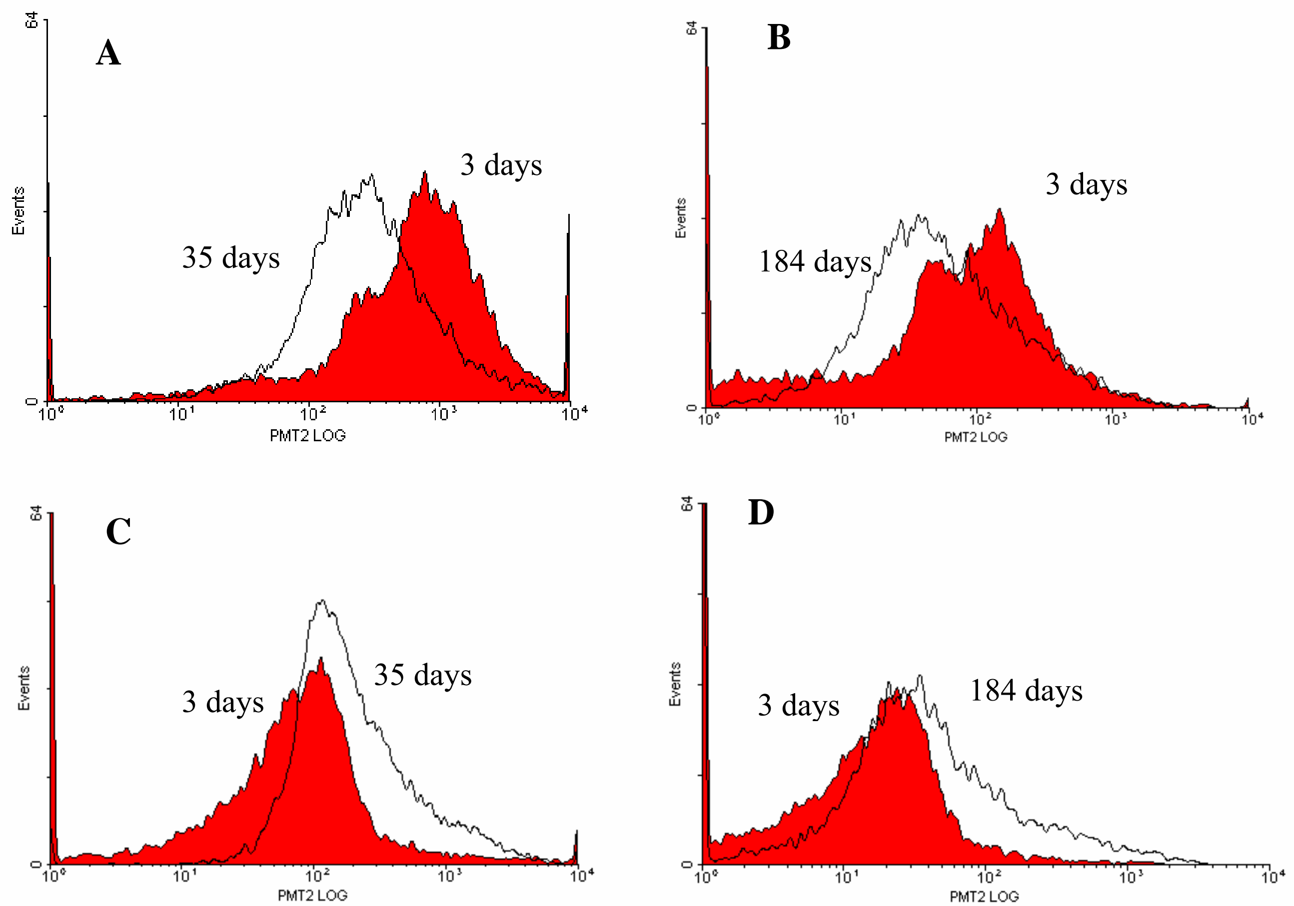


FIG 4.19 Flow cytometric analysis of antigen-antibody interaction.

H. pylori RH 54 cells of various timepoints (3-184 days) were used. A and B show the binding affinity profiles when the anti-spiral IgG was used. C and D show the profiles when anti-coccoid IgG was used. The X-axis measures the intensity of fluorescence and Y axis denotes the cells number.

corresponded with the *26kDa* and *ureA* genes, respectively. Similarly, presence of 2 other virulence genes (*cagA* and *vacA*) was also observed in all cultures (Figure 4.22).

Besides detecting the presence of several important genes, the expression of *26kDa* and *ureA* genes in growing and aging phase cultures were studied. All RNA preparations were treated with DNase I and negative with PCR before performing RT-PCR. The RT-PCR was positive using the *26kDa* and *ureA* primers for the 2 strains at different time intervals (3, 7, 30 and 184/219 days) (Figure 4.23). Results from RT-PCR showed that coccoid forms maintained the *26kDa a*nd *ureA* genes expression throughout the incubation period.

4.6.3 Proteomics analysis

4.6.3.1 Identification of proteins in spiral and coccoid AGE preparations

Eight protein bands (A-H) of the AGE preparations, separated by 1-DE were excised and identified by mass spectrometry. Figure 4.24 depicts the mass and identity of the various proteins. Proteins A and B were identified as serotransferrin precursor and serum albumin precursor, respectively originated from *Equus caballus* (Horse). These proteins are most likely the contaminants from horse serum/ blood incorporated in the medium for culturing *H. pylori*.

The remaining 6 proteins were identified as *H. pylori* proteins. Protein band C of 37.3 kDa which was reduced in the coccoid form was identified as a putative protein, with no apparent function updated. Three proteins which were also present in the coccoid form, though downregulated, were iron superoxide dismutase (Band D), neutrophil activating

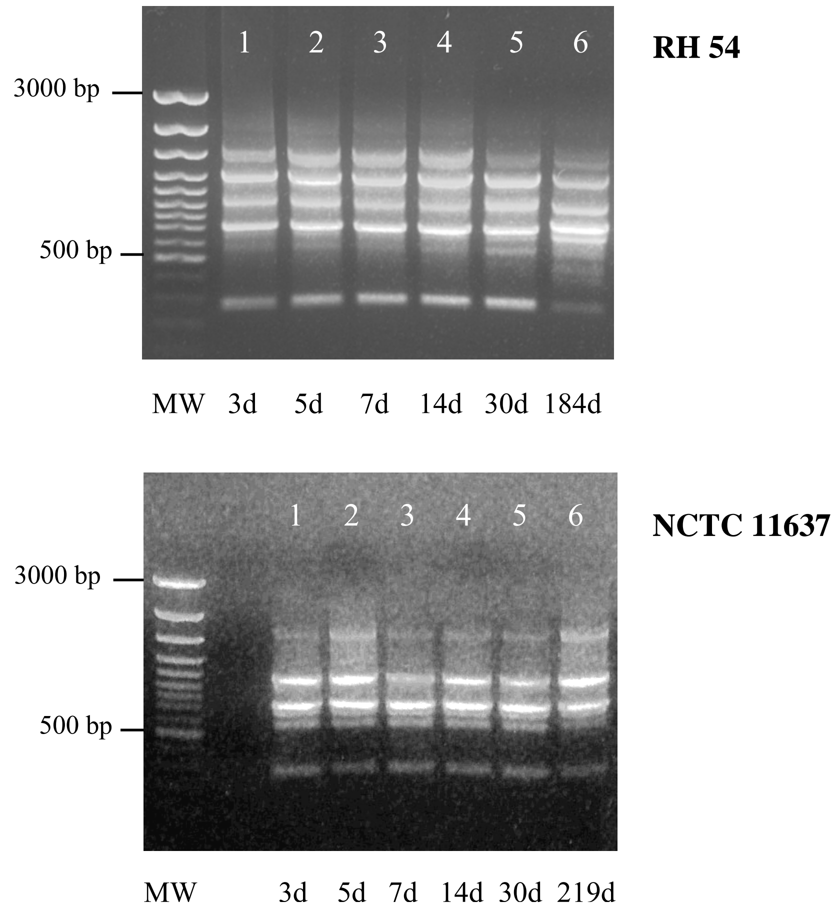


FIG 4.20 RAPD profiles of *H. pylori* cells (RH 54 & NCTC 11637) at various time points.

Lanes 1 through 6 contain cells harvested at various time points (3-219 days). The genomic DNA was conserved during morphological conversion as the DNA profiles of the ageing cultures (coccoid forms) were almost similar to that of the 3-day old cultures (spiral forms). MW shows the DNA size marker (GeneRuler^{TM} 100bp DNA Ladder Plus).

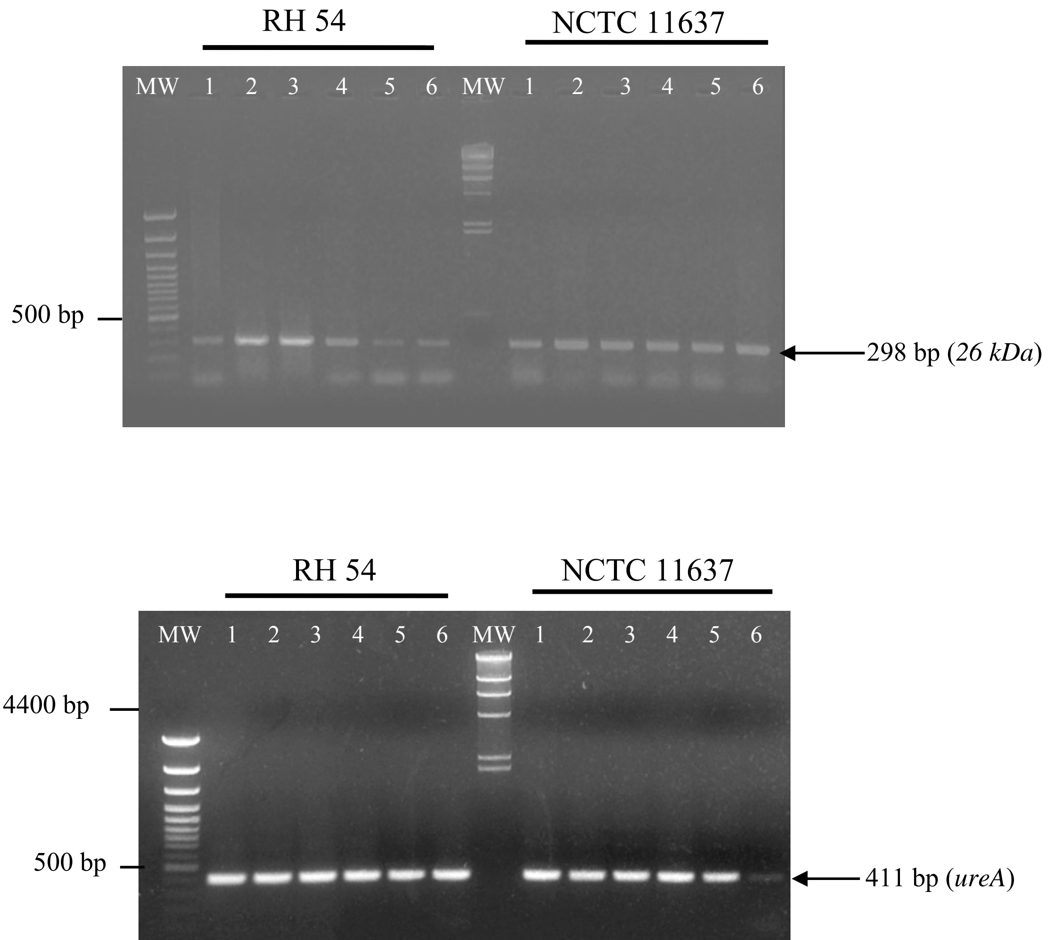


FIG 4.21 PCR analysis of housekeeping genes in *H. pylori* (RH 54 & NCTC 11637) of different time points.

Lanes 1 through 6 contain cells harvested at various time points. For Strain RH 54, Lane 1: 3 day; Lane 2: 5 days; Lane 3: 7 days; Lane 4: 14 days; Lane 5: 30 days; Lane 6: 184 days. For Strain NCTC 11637, Lane 1: 3 day; Lane 2: 5 days; Lane 3: 7 days; Lane 4: 14 days; Lane 5: 30 days; Lane 6: 219 days. MW shows the DNA marker (GeneRuler^{TM} 100bp DNA Ladder Plus/λ DNA digested with *Hin*d III).

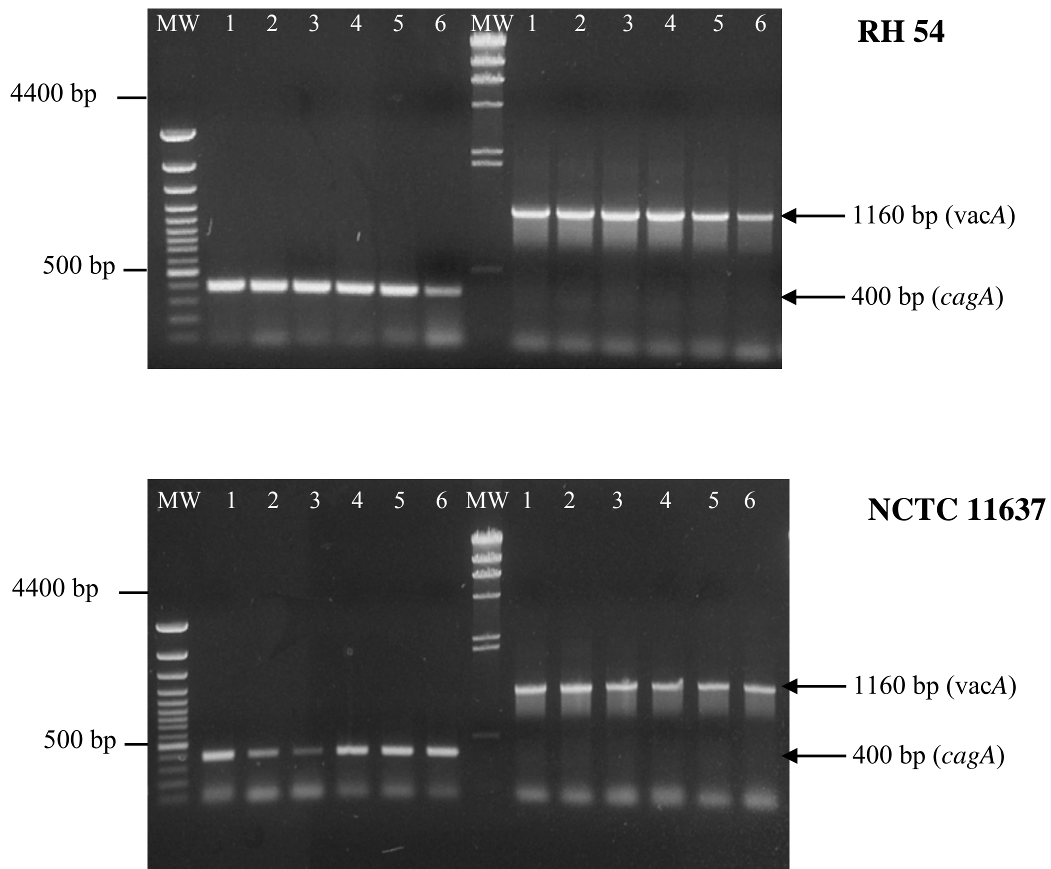


FIG 4.22 PCR analysis of virulence genes in *H. pylori* (RH 54 & NCTC 11637) at different time points.

Lanes 1 through 6 contain cells harvested at various time points. Strain RH 54, Lane 1: 3 day; Lane 2: 5 days; Lane 3: 7 days; Lane 4: 14 days; Lane 5: 30 days; Lane 6: 184 days. Strain NCTC 11637, Lane 1: 3 day; Lane 2: 5 days; Lane 3: 7 days; Lane 4: 14 days; Lane 5: 30 days; Lane 6: 219 days. MW shows the DNA marker (GeneRuler^{TM} 100bp DNA Ladder Plus/ λ DNA digested with *Hin*d III).

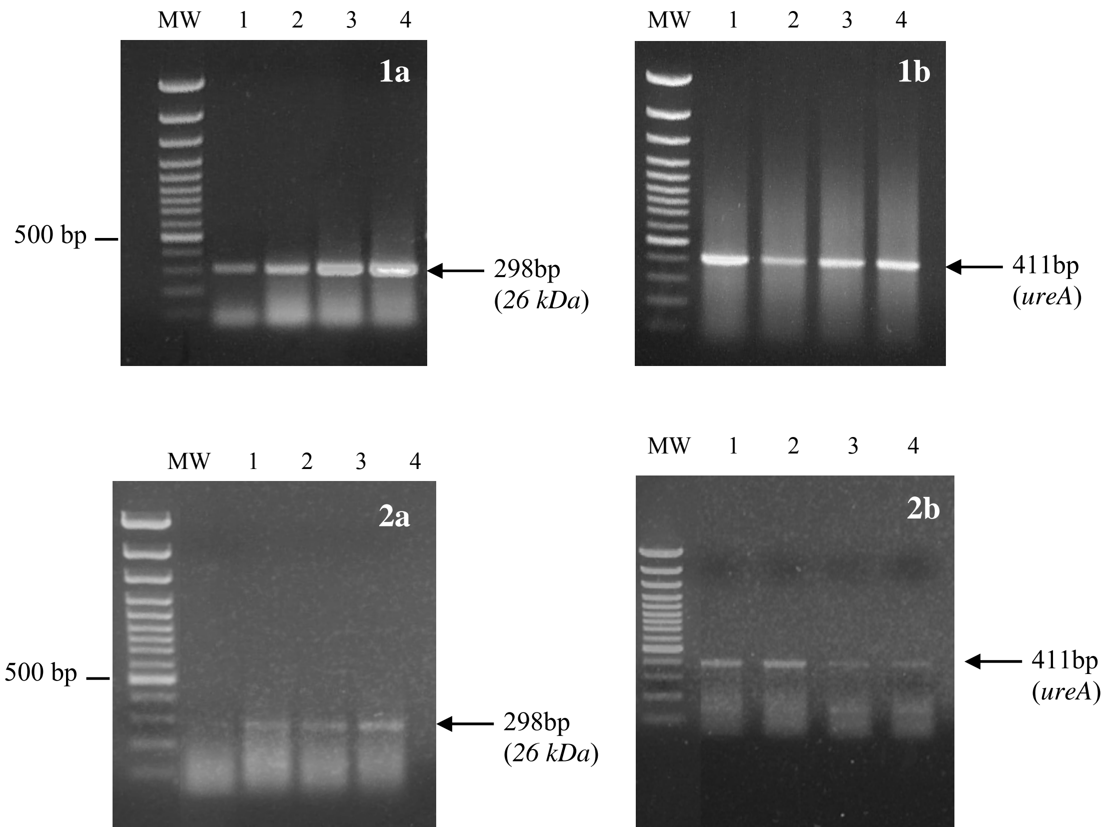
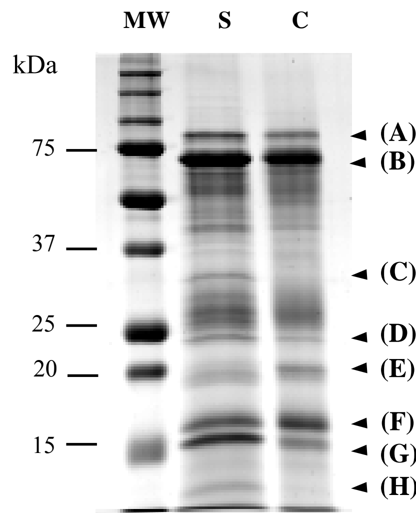


FIG 4.23 RT-PCR analysis of *26 kDa* and *ure A* gene expression.
Agarose gel (1%) electrophoresis of the PCR product of the respective genes showing the detection of mRNA from RH 54 (1a &1b) and NCTC 11637 (2a & 2b). Lanes 1-4 (1a & 1b) represent samples from 3-day, 7-day, 30-day and 184-day cultures, respectively. Lanes 1-4 (2a & 2b) represent samples from 3-day, 7-day, 30-day and 219-day cultures, respectively. MW shows the DNA marker (GeneRuler™ 100bp DNA Ladder Plus).



Band	Protein identity	MW (kDa) database	Functions, if known
A	Serotransferrin precursor (horse)	78.1	
B	Serum albumin precursor (horse)	68.6	
C	Putative	37.3	Conserved with no known function
D	Iron superoxide dismutase	24.5	Destroys radicals which are normally produced within cells & are toxic to the biological systems
E	26 kDa (alkyl hydroxide reductase)	20.2	Reducing a variety of hydroperoxide substrates; essential for growth of bacteria
F	Non-heme iron containing ferritin Pfr	19.4	Iron storage protein
G	Neutrophil activating protein NapA	16.9	May perform analogous functions in iron detoxification & storage
H	Thioredoxin	11.9	Participate in various redox reactions

FIG 4.24 Proteins identified from spiral and coccoid forms of *H. pylori* (AGE preparation.

protein (Band G) and thioredoxin (Band H). As illustrated in Figure 4.24, the *H. pylori* protein E of apparent molecular mass about 20.2 kDa which was upregulated in the coccoid form was identified as the 26 kDa protein. Band F was identified as the non-heme iron containing protein, Pfr. The brief description of the functions of the identified proteins is recorded in Figure 4.24.

4.6.3.2 Comparison of protein profiles in aging cultures - 1D-PAGE

The protein profiles of the aging cultures (RH 54 & NCTC 11637) are illustrated in Figure 4.25. The present study demonstrated that the respective aged cultures (coccoid cells) exhibited highly similar protein pattern as the 3 day old culture (spiral cells) as seen in Figure 4.25. However, the protein bands decreased both in numbers as well as in intensity (down-regulated) during morphological conversion from spiral to coccoid form (Figure 4.25).

4.6.3.3 Comparison of protein maps in aging cultures - 2D-PAGE

Figure 4.26 shows the resolution of proteins from lysed cell pellets of *H. pylori* strain RH 54, at various time points by 2D-PAGE, using a non-linear isoelectric focusing gradient of pH 3-10 and a 12% polyacrylamide gel in second dimension. In all the gels, protein spots are spread over the whole pI range of 3-10 (Non-linear IPG) and the whole MW 10-150 kDa. It was observed that more proteins were resolved at the basic range of the strip. Comparisons of the gels showed that the protein profiles were relatively similar with several main spots found at the same positions. However, a substantial reduction of

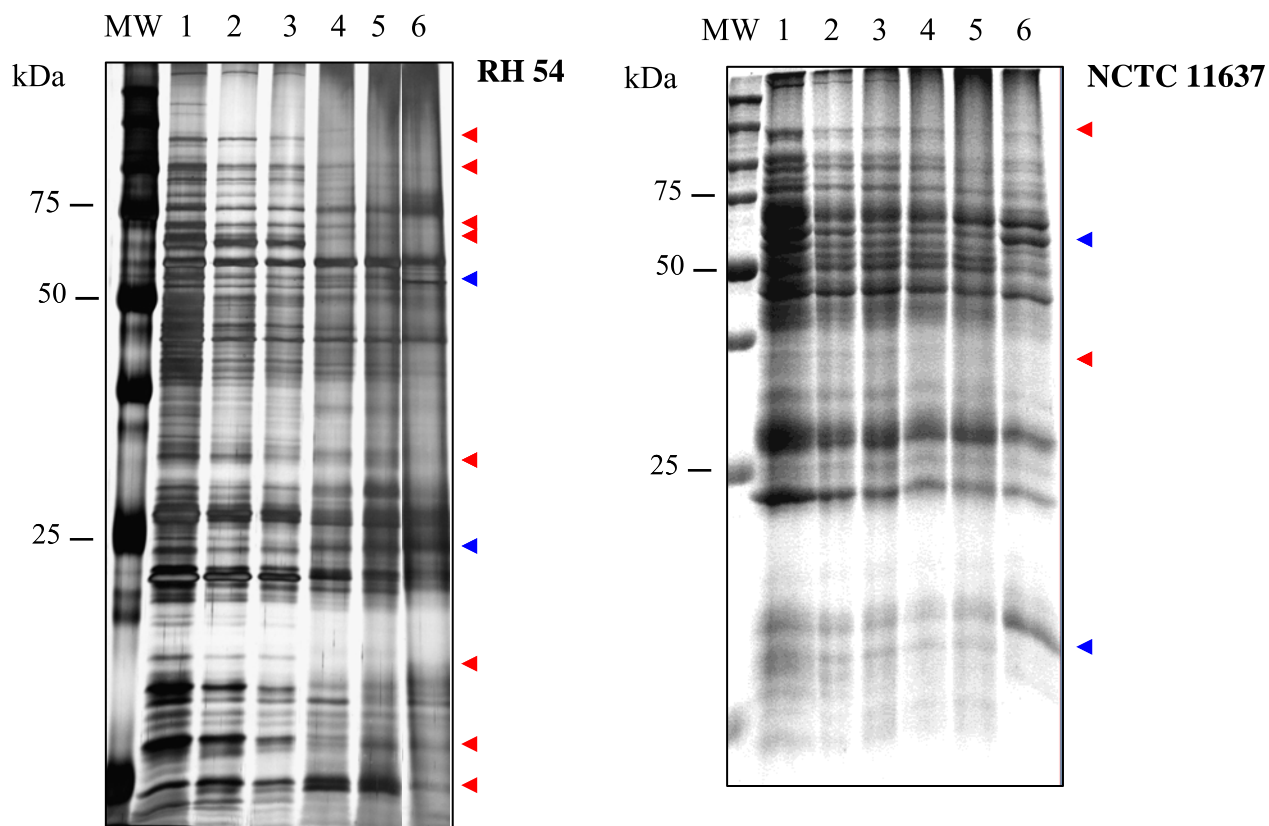


FIG 4.25 Protein profiles of the aging cultures of RH 54 and NCTC 11637.
Decreased in the number of protein bands was observed as the culture aged. Lane 1-5: 3, 5, 7, 14, 30 day old cultures, respectively. Lane 6: 184 day old culture (RH54)/ 219 day old culture (NCTC 11637).
◄ Indicates down-regulated proteins; ◄ Indicates up-regulated proteins in the aged cultures.

the protein spots was observed during the morphological conversion from the spiral form to the coccoid form. In total ~676 spots were detected on the 3 day old *H. pylori* culture (spiral form) as compared to 485 proteins, 374 proteins in 30 and 184 day old cultures (coccoid forms), respectively.

The silver stained gels revealed prominent individual proteins (Figure 4.27). It was also observed that the major proteins each appeared as a "train" of spots (isoforms) migrating at slightly variable *pI* values, a phenomenon commonly ascribes to diverse levels of post-translational modification (Packer *et al.*, 1997).

As the culture aged, horizontal streaks were notably observed in the 2D gels, especially in the 184 day old culture (Figure 4.26F). This might be attributed to the contamination of horse serum proteins/horse blood supplemented to the growth medium or the debris of the growth medium itself. The horizontal streaks were demonstrated to be similar to the gel pattern obtained from resolving proteins from the growth medium which comprised the brain heart infusion supplemented with 10% horse serum (Figure 4.28).

From the stained gels, several protein spots were excised and trypsin digested followed by MALDI-TOF analysis (Figure 4.27). The identities of these spots are summarized in Table 4.12. Comparative analysis showed that protein clusters (Figure 4.27, box j) were reduced significantly in the coccoid cells. Two isolated proteins which formed train of spots on the gels were identified as Urease B (Spot c) and 26 kDa protein (Spot h) and both proteins were down-regulated as compared to the profile obtained from the spiral cells (Figure 4.27 A). In contrast, there were proteins such as g (Translational elongation factor EF-Tu) and i (adhesin thiol peroxidase) which appeared up-regulated in the coccoid cells. Several other proteins identified are VacA (Spot a), Heat shock

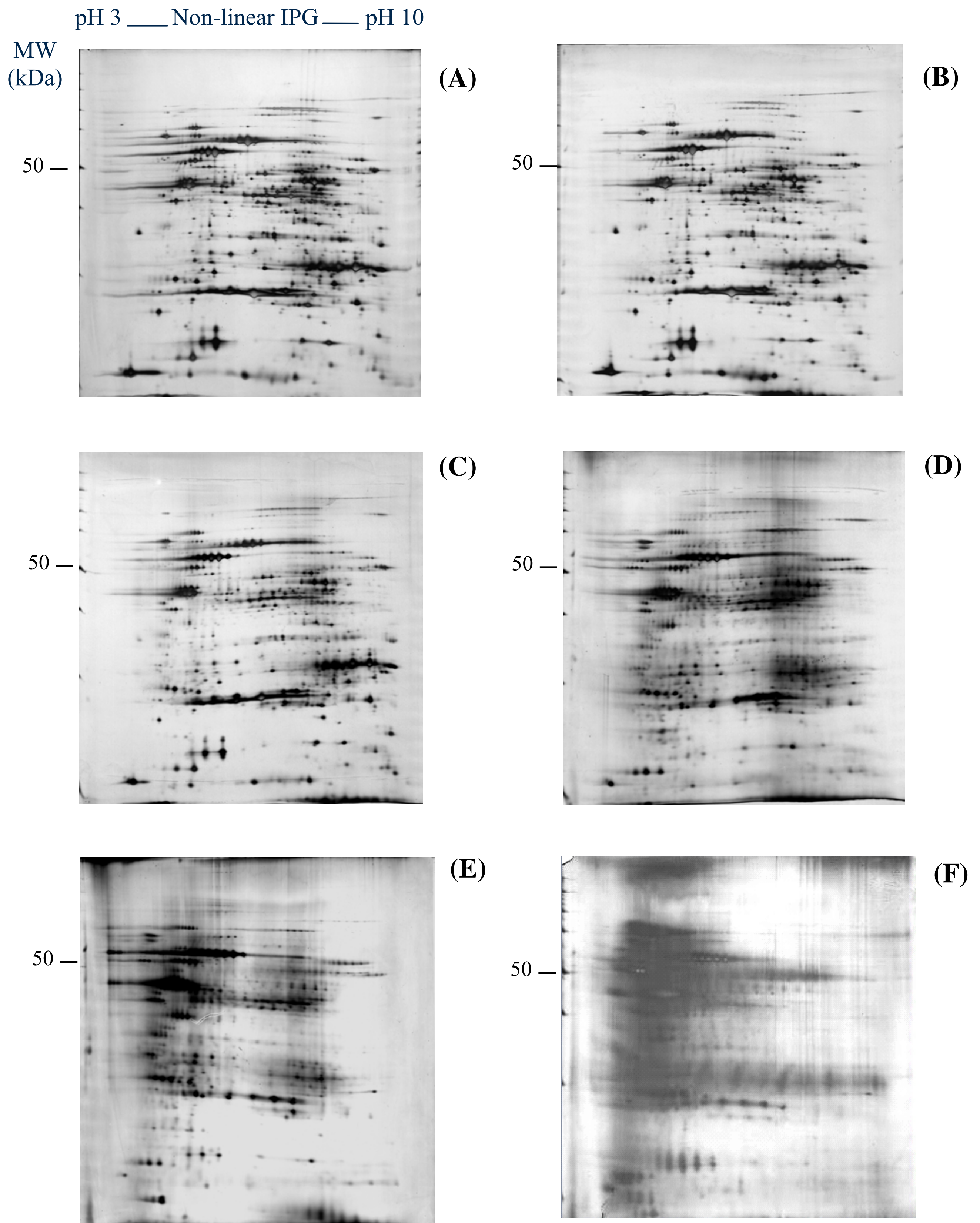


FIG 4.26 2D gels of *H. pylori* RH 54 at various time points.
A: 3 day; B: 5 day; C: 7 day; D:14 day; E: 30 day; F: 184 day

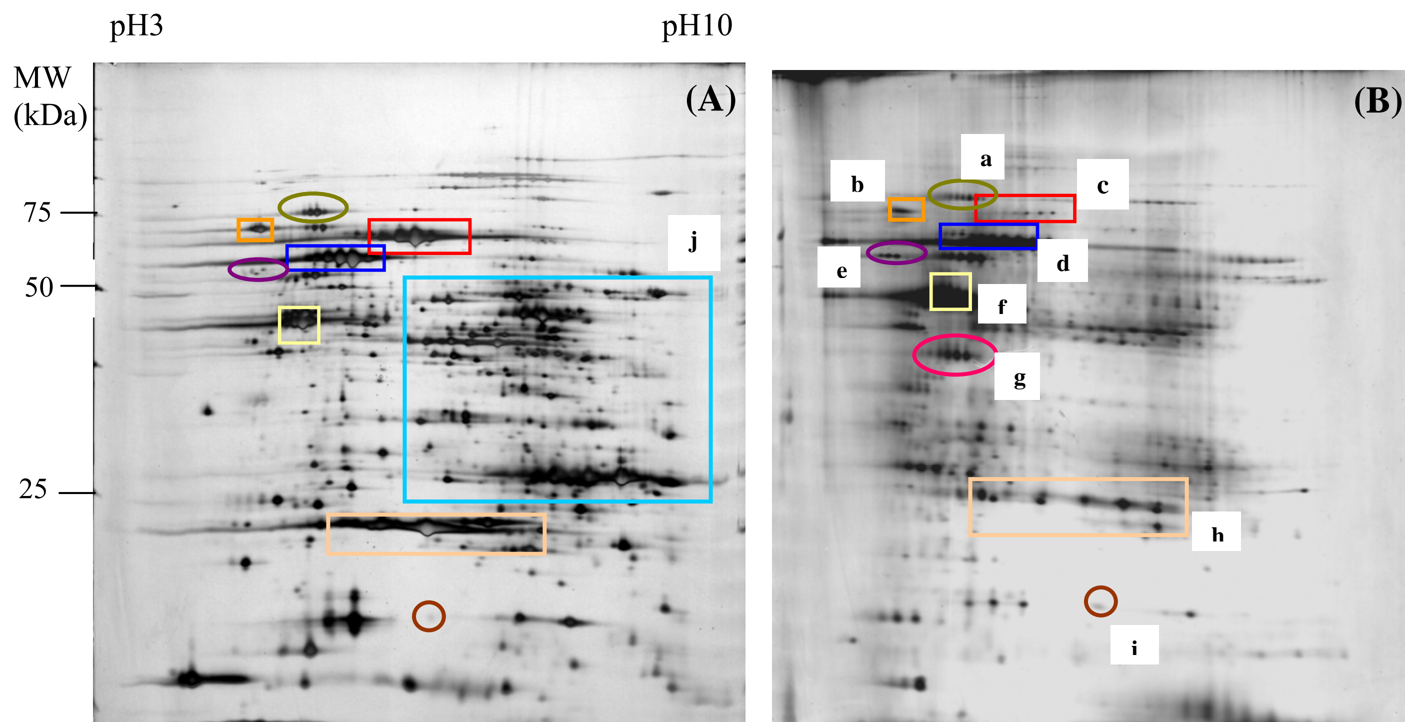


FIG 4.27 2D gels of total cell protein of (A) 3 day old culture and (B) 30 day old culture.

Table 4.12 Identities for isolated protein spots from the 2D gels shown in Figure 4.27

Protein spot	Name	MW (kDa)	pI
a	VacA	93.3	9.34
b	Heat shock protein 70	66.9	5.29
c	Urease B	61.7	5.64
d	Heat shock protein	58.2	5.49
e	Flagellin A	47.6	5.44
f,g	Translational elongation factor EF-Tu (*tufB*)	43.6	5.18
h	26 kDa protein (*tsaA*)	20.2	5.98
i	Adhesin-thiol peroxidase (*tagD*)	18.3	7.69

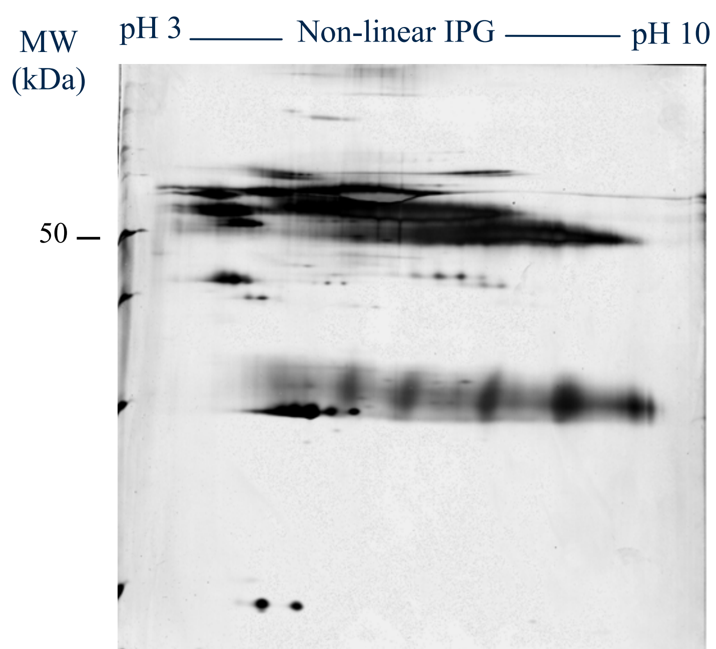


FIG 4.28 2D gel profile of BHI supplemented with horse serum.
Two blotches of protein smear were observed.

proteins (Spot b & d), flagellin A (Spot e) and translational elongation factor EF-Tu (Spot f). These proteins were found to be conserved in the coccoid form.

Comparison of the differential gel profiles has enabled selection of several protein spots of interest for identification. However, due to the detection limit of the coomassie blue staining, low abundance proteins would not be stained. Hence, these spots were not able to be identified. The use of silver staining may alleviate the problem of detection limit. However, the amount of protein presence may not be sufficient for identification by mass spectrometry.

4.6.3.4 2D Differential In-gel Electrophoresis (DIGE)

Differential analysis of the spiral and the coccoid cells was also performed using DIGE, a new emerging technology for proteomic analysis. The proteins from spiral (3 day old culture) and coccoid (30 day old culture) were labelled with Cy3 and Cy5 dyes, respectively. Figure 4.29 A-C illustrates the individual Cy3, Cy5 and the combined gel images. Common proteins present in both morphological forms appeared yellow in the combined image, Figure 4.29C. As this system enables multiplexing of separate protein mixtures on the same gel, it was visibly observed that there was a reduction of proteins in the coccoid cells as compared to the spiral cells. In addition, few coccoid specific spots (appeared red) were observed. However, several proteins were conserved in the coccoid cells as seen in spots in yellow.

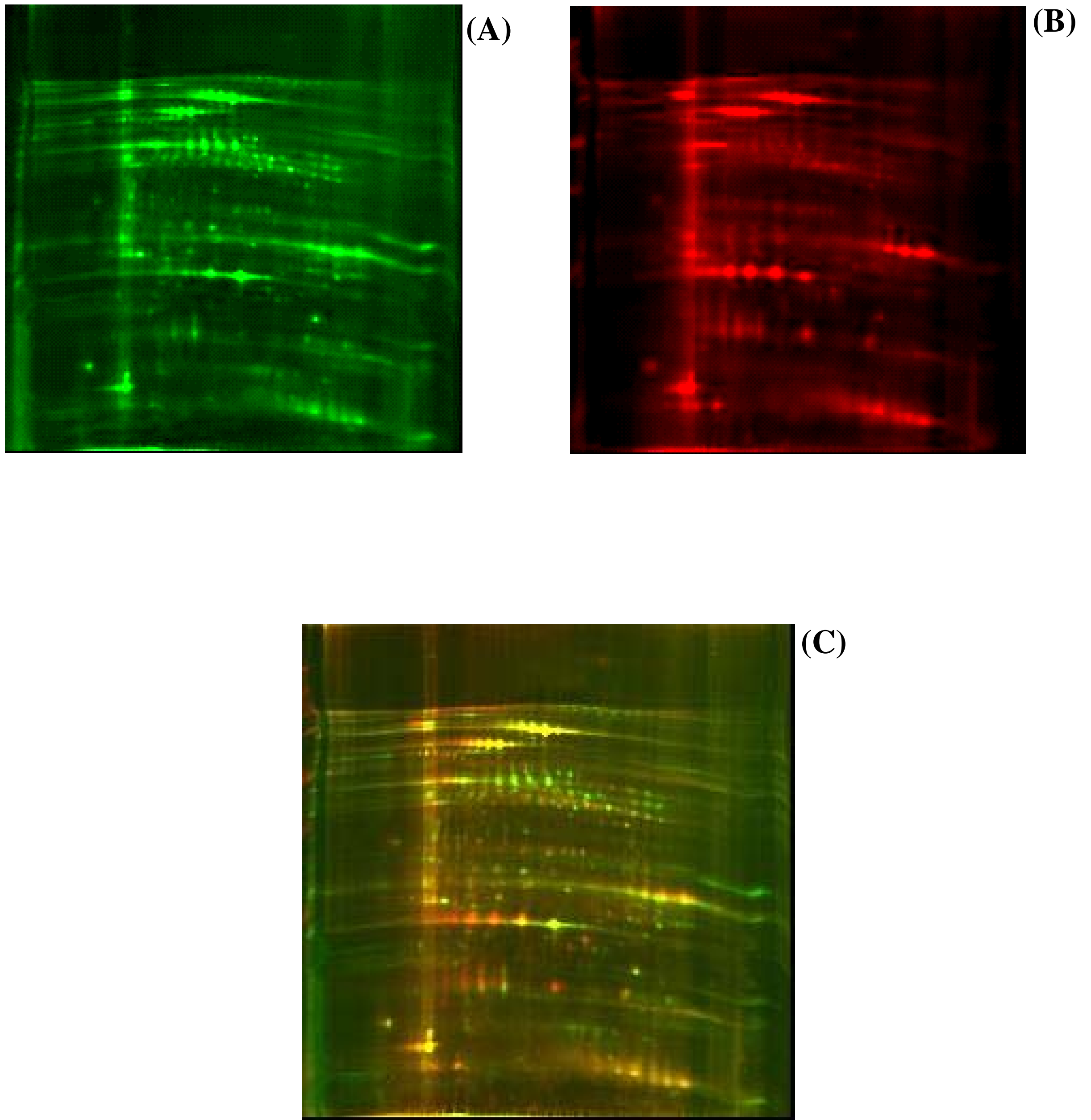


FIG 4.29 DIGE gel images of spiral and coccoid cells of *H. pylori* RH 54.
A: Cy3 image of proteins from 3 day old culture (spiral cells); B: Cy5 image of proteins from 30 day old culture (coccoid cells); C: combined protein profiles (proteins present in both form appeared yellow).

DISCUSSION

5.1 EPIDEMIOLOGY AND TRANSMISSION

5.1.1 Family clusters study-Intrafamilial transmission?

There have been reports addressing the high prevalence of *H. pylori* infection among family members with infected individuals (Oderda *et al.*, 1991; Dominici *et al.*, 1999). Malaty *et al.* (2000) reported a 4-fold increase in prevalence of *H. pylori* infection among children living with positive index mothers compared to children staying with negative index mothers. Interestingly, in the present study of 21 families, at least one young member in each family was seropositive. The possible reason can be attributed to that the children were living with parents and/or grandparents who were seropositive. This observation suggests the possibility of a vertical transmission.

The current study also demonstrates that the rate of seropositivity increases with age; 50% among those with age from 1-15 years old to 100% in subjects aged 61-73 years old (Figure 4.4). This finding is consistent with an earlier report on a larger local population based study of 2,626 individuals (Committee on Epidemic Diseases, 1996). In that study, the rate of seropositivity increased gradually from 3% for children aged 4 years and younger to 71% seropositivity for those aged 65 years and older. Likewise, the observation is seen in accordance with many reports from other countries (Everhart *et al.*, 2000; Malaty *et al.*, 2003).

Western blots analysis

For further evaluation on the intrafamilial acquisition of *H. pylori*, western blotting was performed to investigate the patterns of *H. pylori* antibodies among family members residing in the same household.

Based on the analysis illustrated in Figure 4.5, the western blots profiles detected a range of proteins ranging from ~8 to 130 kDa. In addition, the intensity of the immunoresponse to some antigens varied in each individual. Similar observations were also reported in the study by Pineros *et al.* (2001) who showed that the antigenic components ranged from 30 to 120 kDa. This heterogeneity in the profiles may reflect the diversity at the genomic level (Hua *et al.*, 1998) or the change of immune response to different immunodominant antigens during the course of *H. pylori* infection.

Analysis of the various antibody profiles revealed the presence of the high molecular weight protein, ~120-130 kDa in 98% of the seropositive samples. The molecular weight of the protein points to the CagA protein as described by Covacci *et al.* (1993). Though presence of *cagA* gene related well with peptic ulcer disease (PUD) in the western population, this gene has been reported to be present in 90% of the local population infected with gastroduodenal diseases studied by Zheng *et al.* (2000). Zheng and colleagues (2000) emphasized that *cagA* is not associated with peptic ulcer disease when compared with patients having non-ulcer dyspepsia (NUD). Even though, *cagA* does not serve as the virulence marker for PUD, combined results from current study and that of Zheng *et al.* (2000) illustrated the importance of this high molecular weight protein as a reliable marker for *H. pylori* infection/colonization. In addition, Kuo *et al.* (2003) analyzed the sera for *H. pylori* specific antigens using the commercial kit, HelicoBlot 2.0 (Genelabs Diagnostic, Singapore) and the data showed that the 116 kDa (CagA) protein presented the highest sensitivity. In the same kit, VacA with a MW of 89 kDa was also taken as an important marker for *H. pylori* infection. In the present study, the only

seropositive sample which had no immunoreactive band of 120 kDa, was observed to possess a band of similar MW as VacA.

As shown in Figure 4.5, the medium molecular weight proteins (40-60 kDa) were present in both the seropositive and seronegative samples, including seronegative Family 18. These medium size proteins which parallel subunits of urease, heat shock proteins and flagellins were reported to be responsible for cross-reactivity with antigens of other bacterial species (Andersen & Espersen, 1992; Nilsson *et al.*, 1997). Thus, these groups of proteins are not useful in discriminating *H. pylori* infection.

Family Clustering

Numerous data have been collated to demonstrate familial clustering of *H. pylori* infection (Georgopoulos *et al.*, 1996; Elitsu *et al.*, 1999; Goodman & Correa, 2000). The same strains of *H. pylori* have also been reported to be identified among families based on molecular techniques (Wang *et al.*, 1993; Georgopoulos *et al.*, 1996). In a recent study by Dominici *et al.* (1999) on the general population, they reported that children with both parents who were seropositive had double the risk of being infected by *H. pylori* than those from families of which both parents were seronegative.

In the present study, the antibody profiles of the 72 individuals from 21 families using the western blotting were compared. Based on the Cohen's kappa analysis, 13 families showed certain degree (17%-100%) in agreement of protein patterns among individual household members. Furthermore, parent–child transfer seems to be more frequent. This is shown in the analysis when there were more immunoreactive bands from the parents as compared to the children in the *H. pylori* seropositive families.

Discrepancy in interpretation of the results occurred in 2 families. It was noted that the younger members in Families 6 and 10 remained seronegative despite the seropositivity of the older family members. Family 6 comprised a young couple and a 1-year-old child. The parents were seropositive but the child was seronegative. One plausible explanation could possibly be that there was no seroconversion at the time of this study. As for Family 10, members were of grandparents and grandchildren relationship and results from the blot comparisons demonstrated that only 3 sets of blot patterns demonstrated similar protein profiles for this relationship (Table 4.1). Most studies reported that parent-child and siblings transmission (Rothenbacher *et al.*, 1999; Miyaji *et al.*, 2000; Goodman & Correa, 2000) may play key role in the spread of *H. pylori* infection. In contrast, the study of grandparents-grandchild transfer in *H. pylori* infection has not been explored specifically. Nevertheless, Nwokolo *et al.* (1992) showed that highly similar DNA fingerprints were observed from individuals of 3 generations (grandfather, son and grandson) in the study of a duodenal ulcer disease family. The limited data of the grandparents-grandchild relationship with respect to *H. pylori* infection could likely be the assumption of a less close association between the two generations, possibly due to the wide age gap.

Despite the data from current study supporting a possible vertical familial transfer, there were 5 blot comparisons that showed no similar protein profiles among household members. These subjects could have acquired the infection from other sources besides the family members. Therefore, interfamilial transmission should not be ruled out as a probable mode of acquiring *H. pylori* infection.

Genotyping of the *H. pylori* isolates in families can provide precise information about transmission of specific strain between individuals (Owen & Xerry, 2003). However, no isolates were obtained in the present family cluster study as this study was based on a seroepidemiological survey and no endoscopy was performed. Nevertheless, analysis and results obtained from the present study demonstrate that ELISA and western blotting in combination can also be a useful approach in determining familial status in relation to *H. pylori* infection. The serological analysis in our study is in agreement with the hypothesis of intrafamilial vertical transmission (Taneike *et al.*, 2001; Owen & Xerry, 2003).

In view of this hypothesis, early childhood may be a critical period for the acquisition of *H. pylori* infection. However, how *H. pylori* is transmitted remains elusive as the infective spiral form transforms to the viable but non-culturable coccoid form (VBNC) under various environmental conditions (Nilsson *et al.*, 2002). This inevitably signifies that the coccoid form, which is the morphological form in the extragastric environment, may be a key factor in the transmission cycle of *H. pylori*.

The subsequent serological study was performed to investigate the probable role of the coccoid form encompassing local young symptomatic population.

5.1.2 Prevalence of *H. pylori* infection in children with epigastric pain

Infection by *H. pylori* is likely to be acquired predominantly in childhood (Fiedorek *et al.*, 1991; Boey *et al.*, 1999). A recent study on *H. pylori* status from infancy to adulthood concluded that most newly acquired infection occurred before age 10 (Malaty *et al.*, 2002). Although *H. pylori* infects the younger population, its association

with the development of upper gastrointestinal symptoms such as epigastric pain and pathogenesis of recurrent abdominal pain has been inconsistent (Macarthur *et al.*, 1999; Frank *et al.*, 2000). Findings from a study by Reifen *et al* (1994) demonstrated that *H. pylori* infection in children is not associated with specific clinical symptomatology. In contrast, 2 paediatric studies reported that the epigastric abdominal pain as a clinical presentation occurs more frequently among children with *H. pylori* infection as compared to those without bacterial colonization of the antrum of the stomach (Hardikar *et al.*, 1991; Chong *et al*., 2003). Hence, this study was performed on children with epigastric pain. Children with general abdominal pain were not examined further because there was no clinical indication to justify performing endoscopy or draw blood from this group of patients.

It is also known that a low percentage of paediatric patients with epigastric pain are *H. pylori* positive. This could possibly due to many factors. One of which is that it is the norm for the histopathologists to look for the presence of the spiral form and not the coccoid form of *H. pylori*. If coccoid form was VBNC and had a role in pathogenesis, there is a possibility that the coccoid form will induce host immune response. Therefore, in this present study, we examined the immune response against the spiral-form antigen as well as the coccoid-form antigen in young patients suffering from epigastric pain.

Comparison of seropositivity to spiral and coccoid form antigens

Results from current study show that 7.0% (42/599) of the school children cohort (control group) tested were found to have antibodies against the spiral-form antigen. The finding on non-symptomatic school children (control group) agrees with an earlier study

based on 2626 subjects in various age groups (6 months to older than 65 years), in which 6.7% (48/717) of children younger than age 15 years tested seropositive (Committee on Epidemic Diseases, 1996). However, of the 489 symptomatic children, 13.3% were seropositive with antibodies against *H. pylori* spiral antigen. This is approximately two fold increase in seroprevalence of *H. pylori* infection in children with epigastric pain as compared to the control group using spiral antigen. This rise is significant ($P<0.001$). Interestingly, the rate of seropositivity for antibodies against the coccoid form for the children having epigastric pain and children in the control group were 55.8% and 26.5%, respectively. This observation again depicting a 2-fold increase in the symptomatic patients.

The study shows a 2-fold difference in the rates of seropositivity for the two groups of subjects when either form of the antigen was used in the serological test. However, the differences in the proportions of children within each study group positive between the spiral form and the coccoid form were 4-fold (Table 5.1).

The symptomatic paediatric patients showed increased levels of IgG against both the spiral form and coccoid form of *H. pylori*. The results showed a 4 fold increase in the rate of seropositivity for antibodies against the coccoid-form antigen (55.8%) compared to that for antibodies against spiral-form antigen (13.3%) ($P<0.001$). However, a study by Hua *et al.* (1998a) reported that in the adult symptomatic population, the rates of seropositivity for antibodies against either form were found to be comparable, with 50.7% seropositive for antibodies against the spiral-form antigen and 49.6% against the coccoid–form antigen. More recently, a publication by Figueroa *et al* (2002) also revealed that the sera of 295 infected individuals were highly reactive to both spiral and coccoid forms by

ELISA. The contrast may be due to the possibility that the coccoid form is involved in the initial stage of *H. pylori* infectivity and that there is a relative increase in seropositivity for antibodies against the spiral form with age. This may also explain why we detected a 4 fold increase in the rate of seropositivity for the coccoid form as compared to that for the spiral form in asymptomatic children (Table 5.1) as well as children with epigastric pain. Furthermore, the observed increased prevalence of seropositivity to the coccoid form of *H. pylori* in children with epigastric pain could suggest a possible infective role of the coccoid form.

TABLE 5.1 Comparison of seropositivity of the two differentiated forms between asymptomatic children (controls) and children with epigastric pain

	% Seropositivity	
Antigens	Children with epigastric pain (n=489)	Asymptomatic school children (Control) (n=599)
Spiral form	13.3 [a,c]	7.0 [a,d]
Coccoid form	55.8 [b,c]	26.5 [b,d]

[a,b] Two fold difference between groups when either form of antigens used in ELISA
[c,d] Four fold difference between the 2 antigens within each study population

The present study with children having epigastric pain showed that there is no preponderance of seroprevalence by gender. However, there was an increased seroprevalence with age where the highest seropositivity was among Indian children. This

is in agreement with the findings of Boey *et al.* (1999) on asymptomatic Malaysian children: seropositivity increased with age and most common among Indian children. A study by Kang *et al.* (1997) demonstrated that racial differences in *H. pylori* seroprevalence were more pronounced in the adult population. The study also showed that Indians had the highest prevalence of infection, followed by Chinese and Malays but the Indian subjects had a lower frequency of peptic ulcer than the Chinese subjects.

Association between the spiral and coccoid forms of H. pylori

It was also observed that the coccoid form is strongly associated with the spiral form in the infection process as reported by Chan *et al.* (1994). In this study, it was shown that when IgG antibodies against the spiral form were present, antibodies against the coccoid form were always detected, except for 7 children who were tested seropositive for the spiral form but found to be seronegative for the coccoid form. This observation contributed to the reduced sensitivity (89.2%) of using coccoid antigens in serologic testing when spiral antigen was used as the standard for comparison. However, detailed analysis of the data showed that 6 of these individuals had borderline titre of IgG antibodies against the coccoid-form antigen. The low specificity (49.3%) may mean that there are numerous false positive results. This is expected because, as illustrated in Table 4.5, more than 50% of the test subjects had antibodies against the coccoid form. In view of this observation, the possibility of under-reporting of *H. pylori* infection in this population of young symptomatic subjects must be given due consideration as current tests are designed with reference to the spiral form and not the coccoid form.

The coccoid form is non-culturable *in vitro* and its viability is still subjected to debate. However, in a recent study by Nilsson *et al.* (2002), ATP was detectable for at least 25 days after the morphological conversion from spiral to coccoid form and that mRNA of several genes were present in aged culture. This supports the notion that the coccoid form is probably viable. In addition, a study by Vijayakumari *et al.* (1995) showed that coccoid form of *H. pylori* which adhered to the Kato III cells *in vitro* is similar to the observed interaction of the spiral form with the gastric epithelium *in vivo*. Furthermore, a study using electron microscopy with epithelial cells showed the presence of both spiral and coccoid forms in 3 of the 8 *H. pylori* positive children (Janas *et al.*, 1995). It is therefore probable that the coccoid form, like the spiral form is viable and infective, even though it is non-culturable *in vitro*.

In the present study on young symptomatic children (mean age of 8.5 years) with epigastric pain using *H. pylori* coccoid antigen in the serologic testing, an increased seroprevalence to the coccoid form was noted. It is postulated that the infection may well begin with the colonization of the coccoid form. It is therefore not surprising that most of these symptomatic children were not diagnosed as *H. pylori* positive because usual histological examination and serologic tests used for diagnosis are based on the observation of spiral form.

Whether the coccoid form plays an infective role is not definitive at this stage as coccoid form is being reported as viable but non-culturable (Nilsson *et al.*, 2002). Our findings have provided another facet on the role of the coccoid form of *H. pylori*. This study demonstrates that the effect of the coccoid form has on the immune response in infected individuals, especially children cannot be underestimated. It therefore raises the

question: Is there a possibility of under reporting of *H. pylori* infection in paediatric patients with epigastric pain?

Western blotting – Immunoreactive bands

Immunoblots based on the antibody response to *H. pylori* antigens is a non-invasive assay for diagnosing *H. pylori* infection. Anderson and Espersen (1992) had studied the *H. pylori* infection using Western immunoblot technique and managed to identify several *H. pylori* specific proteins. Recent studies have focused on evaluating the performance of the commercial kits which differentiates the reactivity to each of the multiple *H. pylori* antigens (Park *et al.*, 2002; Kuo *et al.*, 2003; Ogun *et al.*, 2003).

To date, serology studies and commercial kits are based on the antigenic proteins of the spiral form of *H. pylori*. Hence, in this study, western blotting against the spiral forms as well as the coccoid forms was performed on selected sera to examine the immunogenic profiles. The high MW antigen of 144 kDa, presumably the CagA protein (Covacci *et al.,* 1993) was identified by immunoblotting among infected children (Figure 4.10 1b). This observation was also seen in earlier study (Section 4.2) which recorded the presence of a high MW protein (~120 kDa) in 49/50 seropositive family household members. The variability of the size of this protein (116-128 kDa) has been reported by various authors (Cover *et al.*, 1990; Crabtree *et al.*, 1992; Raymond *et al.*, 2000). As in agreement with the study by Covacci *et al.* (1993), this protein which showed up as a minor component in 1-DE (Figure 4.9) is highly immunogenic (Figure 4.10).

Comparing the immunogenic profiles against antigens of the two forms showed that coccoid forms of *H. pylori* are capable of eliciting immune response that is highly similar to that induced by the spiral forms (Figure 4.10).

It was observed from the blot profiles for both antigens that besides the high MW protein (CagA), immunoresponse towards the low MW antigens (13-42 kDa) were characterised predominantly in children with H. pylori infection as compared to those uninfected children (Figure 4.10). Recent studies suggest that the low MW antigens may be associated with *H. pylori* infection (Bode *et al.*, 2002; Kuo *et al.*, 2003). Likewise, an earlier study by Mitchell *et al.* (1996) reported that the initial immunoresponse was directed towards the low MW antigens.

5.2 CLINICAL ISOLATES FROM PAEDIATRIC PATIENTS

In this study, only 15 strains isolated from paediatric patients were used in the characterization study. The low recovery rate of *H. pylori* from biopsy samples could be due to several factors:

(a) the patchy distribution of this bacterium on mucosal surface which could lead to sampling error (Loffeld *et al*., 1991; Nedenskov-Sorensen *et al*., 1991).

(b) delayed in transporting the biopsy samples to the laboratory for processing (Soltesz *et al*., 1992).

(c) in the initial phase of the study, biopsies were not taken from the symptomatic patients.

5.2.1 Genomic fingerprinting- Interpatient and intrapatient variation of *H. pylori*

One of the characteristics of *H. pylori* is the large degree of genetic heterogeneity among the strains. Through the use of DNA fingerprinting methods, this genomic diversity has been engaged in epidemiologic analysis of the mechanism of *H. pylori* transmission (Georgopoulos *et al.*, 1996; Miehlke *et al.,* 1999). Molecular analysis by rRNA gene restriction pattern (ribotyping) of *H. pylori* for 4 members of a Japanese family, performed by Taneike *et al.* (2001), illustrated a familial infection. In the study, the ribotyping pattern of the son who was reinfected after eradication therapy was similar to that of the mother's who did not undergo treatment.

Current study analysed 15 clinical strains isolated from 14 symptomatic children by DNA fingerprinting using RAPD. Of these 15 strains, 2 were isolated from a same patient during re-visit. The DNA fingerprints were shown to be unique to individual patient. Among these, Strains 8 and 9 displayed similar genetic profile as both strains were isolated from the same patient (Figure 4.12). In an earlier study by Hua *et al.* (1999) which the *in vitro* interaction of 2 *H. pylori* strains in a mixed growth was assessed. In their study, they observed the growth of a predominant strain. Consequently, our data suggests that each patient was infected with a genetically predominant strain of *H. pylori*.

To further confirm that a single strain predominates in the gastric antrum of an individual, 5 strains were randomly selected for the clonal study. From each of these 5 selected strains, a total of 5 colonies were randomly chosen as clonal representatives for DNA fingerprinting. The genetic profiles from the 5 clones of a single isolate were indistinguishable. A similar observation of a homogenous population of *H. pylori* was also noted by Marshall *et al.* (1995).

5.2.2 *H. pylori* genotypes in children with epigastric pain

H. pylori infection is one of the commonest chronic bacterial infections in human. However, only approximately 15-20% of *H. pylori* infected individuals will develop gastroduodenal disease (Suerbaum & Michetti, 2002). Presently, even with the phenomenal progress in research on *H. pylori*, the pathophysiological role of this human gastric pathogen remains to be resolved, particularly in the younger population. Despite the high degree of genetic heterogeneity among *H. pylori* isolates (Akopyanz *et al.*, 1992; Mukhopadhyay *et al.,* 2000), several studies have reported that specific genotypes of *H. pylori* increased the risk of a clinically significant outcome (Blaser *et al.*, 1995; Atherton *et al.*, 1997; van Doorn *et al.*, 1998).

At present, several virulence associated genes have been identified in genome of *H. pylori*: *cagA*, *cagE*, *vacA*, *iceA* and *babA2* (van Doorn *et al*., 1998; Gerhard *et al*., 1999; Fukuta *et al*., 2002). Among these, *cagA* gene is a widely studied virulence factor. The *cagA* gene is located at the most downstream of the *cag* pathogenicity island (PAI) and encodes for a highly immunogenic outer membrane protein, CagA (Covaci *et al*, 1993). In several studies, *cagA* gene/CagA protein is shown to be associated with a more severe clinical outcome in both adult and children (Blaser *et al.*, 1995; Queiroz *et al.*, 2000; Yahav *et al.*, 2000). However, this observation is inconsistent as geographical differences have shown to be otherwise. Nearly all East Asian strains are *cagA* positive, independent of clinical manifestations (Pan *et al.*, 1997; Maeda *et al.*, 1998, Zheng *et al.*, 2000). In a recent study by Day *et al.* (2000), another gene in the *cag* PAI, *cagE* was shown to be associated with duodenal ulceration in children. In current study, the 15 clinical isolates from children with epigastric pain were examined for the prevalence of

both *cagA* and *cagE* status. Results showed that all the 15 isolates were positive for both genes. The finding is in accordance with earlier studies by our research team, which also reported a high prevalence of *cagA* in the local adult population (Hua *et al.*, 2000b; Zheng *et al.*, 2000). Hence, present study supports that *cagA* is not a useful virulence marker for peptic ulcer disease (Park *et al.*, 1998; Perng *et al.*, 2003; Qiao *et al.*, 2003), at least in the Asian population. Similarly, the prevalence of *cagE* is also high in our region irrespective of clinical status. Fukuta and colleagues (2002) provided added data that *cagE* will not serve as an invariable marker for peptic ulcer disease as their study has failed to show an association between *cagE* gene and duodenal ulcer in 2 areas in Japan.

Another important virulence determinant is VacA, which induces the formation of intracellular vacuoles in eukaryotic cells *in vitro* (Cover, 1996; Harris *et al.*, 1996). The *vacA* gene contains 2 distinct regions: the signal region (s) and the middle region (m). The signal and middle sequences could be designated s1 (subtypes s1a, s1b and s1c) or s2 and m1 or m2, for s and m regions, respectively (Yamaoka *et al.*, 1999). The s1 and m1 subtypes have been linked to more severe clinical manifestations (Basso *et al.*, 1998; De Gusmao, 2000). In a study with Taiwanese patients, Wang and colleagues (1998) typed the *H. pylori* isolates with the different *vacA* genotypes using primers adopted from Atherton *et al.* (1997) and their modified primers for m1T and m1Tm2 types. Interestingly, the results suggested that s1a/m1T strains were associated with higher ulcer prevalence. In the present study, we examined the distribution of the *vacA* genotypes in 15 *H. pylori* paediatric isolates and identified 6 different genotypes (Table 4.9). In this study, the commonly observed genotype is the s1c/m2. This genotype is also reported in the study by Yamaoka *et al*. (1999), which showed that this genotype was predominant in

Japan and Korea. The s1c allele, which is the predominant *vacA* genotype in Japan and East Asia (Wong *et al.*, 2001) was also observed in high incidence in the present study. In contrast, *vacA* s1b and s2 were rare. This observation is contrary to the Brazillian study (De Gusmao, 2000) which reported the association of s1 allele with duodenal ulcer in children and most of the s1 strains (97.5%) had the s1b allele. However, s1b allele was not present in our local isolates.

A novel gene, *iceA* (induced by contact with epithelium) has 2 allelic variants, namely *iceA1* and *iceA2*. The function of this gene is not defined but there is significant homology to a type II restriction endonuclease. The present study demonstrated *iceA1* allele to be the predominant subtype in local isolates. This is not surprising since high incidence of the *iceA1* allele has been reported as the predominant subtype in East Asia while *iceA2* is the predominant allele in the USA and Columbia (Yamaoka *et al.*, 1999).

Besides examining the prevalence of several important virulence factors, the 15 strains were also typed for *babA2*. This gene encodes the complete adhesin which has been shown to mediate adherence of the bacteria to human Le^b blood group antigens on gastric epithelial cells (Ilver *et al.*, 1998). Studies based on Western population suggested an association between *babA2* positive status with duodenal ulcer as well as gastric carcinoma (Gerhard *et al.*, 1999; Oliveira *et al.*, 2003). In contrast, several studies have shown that the circulating *H. pylori* strains in the Asian countries are *babA2* positive, regardless whether they were isolated from asymptomatic or diseased patients (Kim *et al.*, 2001; Mizushima *et al.*, 2001; Lai *et al.*, 2002). Our findings from current study showed that 80% of the local paediatric isolates studied possessed the *babA2* gene and this is in agreement with those studies from the Asian countries.

Taken together, the results from this paediatric population study and an earlier adult population study (Zheng *et al.*, 2000), neither *babA2* nor combination of *cagA*, *vacA* and *iceA* would be useful markers to identify patients who are at higher risk for specific *H. pylori*-related diseases in this geographic region. Furthermore, present study adds to the evidence that demonstrates *H. pylori* genotypes in Western and Asian countries are distinctly different.

The current study also demonstrates that co-infection with multiple *H. pylori* strains is uncommon in Singapore because the 5 individual clones selected from each of the 5 different strains showed similar RAPD profiles representing a single genotype. This finding is unlike several reports that described multiple-strain infection (Yakoob *et al*., 2001; Ashour *et al*., 2002). One of the studies was by Morales-Espinosa and colleagues (1999) which detected 2 or more different *vacA* genotypes in 17/20 patients. However, our results support the finding that a predominant strain exists in each patient (Hua *et al*., 1999a).

5.2.3 Antibiotic resistant *H. pylori* in paediatric patients

The National Institutes of Health Consensus Development Conference has recommended the addition of antimicrobial agents to anti-secretory drugs for the treatment of patients with *H. pylori* associated peptic ulcer disease (NIH, 1994). Hence, the first-line therapy regimen widely used today is the combination therapy with 2 antibiotics and bismuth or an acid pump inhibitor (Fraser *et al.*, 1999; Gold *et al.*, 2000; Bazzoli *et al.*, 2002). The antimicrobial agents frequently used in the treatment regimens include amoxicillin, tetracycline, clarithromycin and metronidazole (Unge, 1998).

Primary resistance to nitroimidazoles and macrolides in *H. pylori* has been described in numerous studies and resistance of the bacterium to these antibiotics has been a major cause for therapeutic failure (Adamek *et al.*, 1999; Pilloto *et al.*, 2000; Perez *et al.*, 2002). As a result, the resistance of *H. pylori* to the available antibiotic treatment regimens has been a growing problem. In developed countries, metronidazole resistance is observed to range 10 to 50% in adults infected with *H. pylori* (Graham, 1998; Adamek *et al.*, 1999). On the other hand, the rates of clarithromycin resistance are relatively low but rate has been increasing in recent years (Crone *et al*., 2003; Loffeld & Fijen, 2003). As in local context, our research team has documented earlier, a 4 years period of investigation on *H. pylori* resistance to metronidazole and clarithromycin in the adult population (Hua *et al.*, 2000a). In the study, the rate of metronidazole resistance increased over the 4 years from 20% in 1995 to 47% in 1998 and was believed to have reached a platform of ~50%. However, clarithromycin resistance was observed to be comparatively low (6%) in the study population (Table 5.2).

The incidence of antimicrobial resistance to *H. pylori* in the local young children with *H. pylori* infection has yet to be investigated. Hence, this study provides a preliminary data regarding resistance rates of *H. pylori* isolates from local symptomatic children. The antibiotic sensitivity tests showed a high rate of resistance of *H. pylori* to metronidazole (53.3%) and moderate resistance to clarithromycin (13.3%) but no resistance to amoxicillin or tetracycline has been detected. These results are in agreement with a recent study by Rerksuppaphol *et al.* (2003) which reported the resistance rate to metronidazole and clarithromycin to be 43.5% and 8.7%, respectively. Likewise in their

study, all the strains were sensitive to amoxicillin and tetracycline. Table 5.2 depicts the high prevalence of metronidazole resistant *H. pylori* in our region.

TABLE 5.2 Comparison of antibiotic resistance in the adult and children populations

	Adult population[1] (Resistance isolates/total isolates)	Children population[2] (Resistance isolates/total isolates)	*p* value
Clarithromycin resistance	18/282 (6.4%)	2/15 (13.3%)	1.1
Metronidazole resistance	130/282 (46.1%)	8/15 (53.3%)	0.3

1 Hua *et al*., 2000

2 Data from present study

The high resistance of *H. pylori* to metronidazole could have been linked to the usage of this drug in the community. Metronidazole is used for treatment of parasitic infections, dental and gynaecological infections. The idea that the resistance of *H. pylori* to metronidazole for treatment of unrelated infection such as gynaecological infections is not valid for children. However, the history of using metronidazole in treating infectious disease other than *H. pylori* probably exposed the children to sublethal concentrations of the drug and may select for resistant strains or augment the development of resistance in previous sensitive strains. This hypothesis is enhanced when a recent publication by Lui *et al.*, 2003 has demonstrated that in the local adult population, the rate of metronidazole

resistance was found to be more prevalent in non-ulcer dyspepsia than in peptic ulcer patients.

There is a need for constant surveillance of both prevalence and evolution of antibiotic resistant *H. pylori* in children because of the association of the infection with prolonged or even lifelong risks of gastroduodenal diseases during adulthood. As the number of isolates obtained in this study is small, current study serves only as a preliminary documentation of antibiotic resistance in *H. pylori* isolates from children with epigastric pain *per se*. In order to arrive at a significant conclusion regarding the rate of antibiotic resistance in our local paediatric population, a substantial number of *H. pylori* isolates is needed. Subsequently, the collated findings can contribute to the enhancement of the eradication of *H. pylori* by providing guidelines for therapeutic recommendations.

5.2.4 Lewis antigens expression in clinical *H. pylori* isolates

H. pylori has been reported to express fucosyl-transferases which enable the bacteria to synthesize Lewis (Le) antigens (Appelmelk *et al.*, 1996). Serotyping of clinical strains of *H. pylori* based on the occurrence of Le epitopes was evaluated in the study by Simoons–Smit *et al* (1996) and had shown differentiation between strains. It has been documented that the type II carbohydrate antigens, Le^X and Le^y were expressed by > 80% of *H. pylori* isolates from symptomatic patients (Simoons–Smit *et al.,* 1996; Wirth *et al.*, 1997). Hence, in this study, the *H. pylori* strains isolated from children with epigastric pain were characterized by their Le antigens expression.

By ELISA, it was demonstrated that Le^X and Le^y were frequently expressed by local *H. pylori* isolates from children having epigastric pain. This observation also holds

true for the isolates from local adult patients as reported in the study by Zheng and colleagues (2000).

Le^X was shown to play a role in colonization via a Lewis antigen mediated adherence process as demonstrated in a series of studies (Edwards *et al.*, 2000; Heneghan *et al.*, *2000*). Edwards and colleagues showed that in contrast to the parental strain (Le^X positive strain), 2 mutant strains which were unable to produce Le^X did not adhere to the epithelial cells of gastric pits of human antral tissue sections. A recent study by Munoz *et al.* (2001) which compared the expression of Le^X and Le^Y in *H. pylori* isolated from children and adults showed that Le^X expression was significantly higher in clones from children than in clones from adults. They further commented that by expressing Le^X during early phase of the colonization, *H. pylori* might have escaped the immune response that otherwise would have eliminated the infection. This postulation was put forward because Le^X found on the surface of the eggs of parasitic worm *Schistosoma mansonii* were able to down-regulate the T lymphocytes responses during an infection (Velupillai *et al.*, 1994). Therefore, there is a likelihood that *H. pylori* Le^X may shift the immunity from cell-mediated to that of antibody-mediated which as in *H. pylori* is ineffective in eliminating the infection of the bacteria by the host. A high frequency of Le^X expression in all 15 *H. pylori* isolates from infected children was observed in the present study, agreeing with the report by Munoz *et al* (2001) which also demonstrated a high Le^X expression in children. Moreover, the study by Zheng *et al.* (2000) with the local adult population showed that there was no significant difference between non-ulcer dyspepsia and peptic ulcer patients for Le^X expression. Hence, the findings might infer that Le^X may possibly play a part in the early stage of infection possibly by assisting colonization.

Some *H. pylori* strains expressed the type I Lewis antigens, Le^a and Le^b. Current study showed that 6.7% (1/15) and 20% (3/15) of the isolates tested expressed Le^a and Le^b, respectively. This frequency is noted to be higher than the study by Rasko *et al.* (2001) which showed that only 4.8% of the isolates from symptomatic patients expressed the type I Lewis antigens. The difference in the frequency of expression could be due to the populations studied. It is interesting to note that Chinese patients expressed Le^a and Le^b more frequently as compared to strains isolated in the western population (Zheng *et al.*, 2000) while Chinese expresses the Le(a+b+) phenotype more often as compared to the caucasians (Broadberry & Lin-Chu, 1991). This observation holds as 3 out of the 4 isolates in our study which expressed Le^a or Le^b were from the Chinese ethnicity.

5.3 LEWIS ANTIGENS EXPRESSION OF *H. pylori* DURING MORPHOLOGICAL CONVERSION

The ability of many *H. pylori* strains expressing Le antigens (Le^a, Le^b, Le^X, Le^y) similar to those of the host is considered by many to be a major factor in the ability of this gastric organism to cause chronic gastroduodenal diseases (Applemelk *et al.*, 1996; Moran *et al.*, 1996; Zheng *et al.*, 2000). In view of this characteristic of the bacteria and with the results obtained earlier regarding coccoid form being a probable infectious form (Section 4.3), it was decided to examine the Le antigens expression during *H. pylori* morphological conversion from the spiral to coccoid form.

Results from the present study show that *H. pylori* retained Le antigen expression even after prolonged incubation (Figure 4.17). Additionally, a general trend was observed when assessing the level of Le antigens expression at various time points of the 2 strains

(RH 54 & NCTC 11637) is that both strains exhibited peak expression at 5th & 30th day of growth. As illustrated in Figure 4.17, the Le antigens expression increases substantially from day 3 to day 5 of growth. This phenomenon could be attributed to the actively dividing bacteria cells (spiral form) at this phase of growth and hence an increased in Le expression. Mimicry of human Le antigens by *H. pylori* has been suggested to be a mechanism of camouflage to escape elimination by host immune system (Wirth *et al.*, 1997) or to facilitate adherence of the bacteria to the gastric mucosa (Edwards *et al.*, 2000; Moran *et al.*, 2000). If the postulation was valid, then an increase in the amount of Le antigens expressed as seen in this *in vitro* study would probably provide an added advantage to the bacteria in the initial infection.

Following the peak at 5th day of culture, Le antigens expression started to decline until 15th day of incubation. After which, the expression was seen to increase again. The period of 5th to 15th day of culture is probably the period of morphological conversion where coccoid forms became predominant (Benaissa *et al.*, 1996). Previous studies have demonstrated that the membrane of the *H. pylori* cells changed upon entry into the coccoid state (Benaissa *et al.*, 1996; Sorberg *et al.*, 1996). It may mean that the decrease in the Le antigens expression is related to these membrane alterations, thus resulting in a loss of membrane integrity. The study by Rasko *et al* (2001) further supports the relationship of declining Le expression with membrane modifications as Le antigens were observed to shed into the supernatant during the stationary growth phase of *H. pylori*. This observation could imply that in the *in vivo* scenario, shedding of the Le antigens may function as an immunological decoy, thus preventing the coccoid form from eliminated by the host response.

It is indeed interesting to note the registering of another peak of Le antigen expression after a prolonged incubation for 30 days. One would assume that at this time point, all the cells would have converted to the coccoid form. This observation suggests that the coccoid form is capable of expressing Le antigens and probably not likely to be the degenerate form as reported by Kusters *et al.* (1997). This then raises the question if the coccoid form is the most infectious at this time point (30 days old culture). Further assessment of the Le antigens expression beyond 30 days of incubation and *in vivo* study with the 30 days old coccoid form may contribute to our understanding of the variation of Le antigens expression during *H. pylori* morphological conversion.

5.4 IMMUNOELECTRON MICROSCOPY & FLOW CYTOMETRIC ANALYSIS

The immunoelectron micrograph (Figure 4.18) shows that the AGE antigens of *H. pylori* used for the serology in this study consisted of surface bound proteins. This is apparent as clusters of gold particles were observed to localize on the surface of the whole bacteria. The 3 day old cells were subjected to both the spiral and coccoid antibodies which were raised against the spiral and coccoid *H. pylori* cells, respectively. If the spiral form of *H. pylori* expressed spiral specific antigens and *vice versa* for the coccoid form of *H. pylori*, one would probably perceive a higher affinity of the spiral antibodies to the spiral cells as compared to the coccoid antibodies. However, this phenomenon was not observed under the immunoelectron microscopy. There is no apparent difference in the density of gold particles observed around the cells with either antibodies as illustrated in Figure 4.18A & B. In view that the antigens used were basically localized on the surface

of *H. pylori* (Figure 4.18), evaluation of the antigen-antibody interaction was carried out using flow cytometry. This is a sensitive method that allows rapid mutliparametric analysis (Blom *et al.*, 2001). By using flow cytometry, it was shown that though the spiral and coccoid antigens shared many common antigens (overlapping area in Figure 4.19), it is still probable that the spiral/coccoid specific immunogenic proteins do exist.

As seen in Figure 4.19A & B, the spiral antibodies showed preferred affinity for the 3 day old *H. pylori* spiral cells. Whilst, when the coccoid antibody was used, there was a shift of the fluorescence to, indicating the preference for the coccoid cells (30 and 184 day old) (Figure 4.19). These observations affirmed the use of the coccoid antigens in the earlier serology tests to detect patients who could have been infected with the coccoid form of *H. pylori*.

5.5 GENOMIC STUDY OF AGING *H. pylori* CULTURES

In order to understand the changes reflected during the morphological conversion, the integrity of the nucleic acids which contains the information for cellular multiplication (Moat & Foster, 1988) in both the morphological forms of *H. pylori* was examined. It was shown that the RAPD profiles of different aged cultures displayed highly similar patterns (Figure 4.12). Furthermore, the PCR assays detected the presence of the gene for a 26 kDa protein (*tsaA*), *ureA* and two important virulence genes (*cagA* & *vacA*). This was demonstrated even in cultures which were more than 6 months old (184/219 days). These observations demonstrated no evidence of DNA fragmentation after prolonged incubation as judged by the RAPD profiling and PCR amplification of the *H. pylori* specific genes during conversion of the spiral to coccoid form. In the study by Ren *et al.* (1999), they too

observed genes coding for *ureC*, *cagA*, 26kDa and *16S* rRNA that remained intact in both the helical and coccoid forms of *H. pylori*.

Besides demonstrating the integrity of the DNA, it was also observed that the aged culture maintained the expression of *26kDa* and *ureA* genes (Figure 4.23) throughout the incubation period. Several studies have also reported expression of virulence genes during conversion to the coccoid form. One of these studies is a report from Sisto and co-workers (2000). They showed that in some *H. pylori* strains, the *ureA*, *cagA* and *vacA* genes were still expressed after 31 days of prolonged incubation. Similarly, in another study (Nilsson *et al.*, 2002), the expression of mRNA for *vacA*, *ureA* and *tsaA* (26kDa) were detected in 28 months old non-culturable cold-starved cells suspensions of *H. pylori*. In addition, the cellular ATP was detectable for at least 25 days after the morphology conversion (Nilsson *et al.*, 2002).

The role of coccoid form of *H. pylori* remains an open topic for debate. The coccoid form of some organisms, such as *Vibio vulnificus* (Oliver *et al.*, 1995) was able to recover from the VBNC state by a temperature upshift. However, to date, resuscitation of VBNC cells of *H. pylori* has been futile. Some authors suggest that the coccoid morphology, which is generally non-culturable is merely dead cell (Kusters *et al.*, 1997). Whilst others are in agreement that a small proportion of the coccoid cells are viable as they maintained the cellular functions such as DNA replication, transcription and translation (Mizoguchi *et a*l,. 1999; Zheng *et al.,* 1999; Nilsson *et al.*, 2002). Results from our study is in support of the notion that the coccoid form of *H. pylori* is viable as the chromosomal integrity was maintained and basic cell functions such as transcription and translation were also demonstrated.

5.6 PROTEOMIC STUDY OF AGING *H. pylori* CULTURES

5.6.1 Proteins identified from 1D-PAGE

As discussed in the earlier sections, the present study shows that the coccoid form of *H. pylori* could be alive in the dormant state. The final section of this study aims to examine the viability of the coccoid form by evaluating its protein synthesis.

The 1D PAGE protein profiles for the AGE preparations (Figure 4.24) and SDS-PAGE for the time course experiments (Figure 4.25) show that the protein patterns of the coccoid populations are relatively similar to that of the spiral forms. However, as expectedly, the protein synthesis in the coccoids is relatively reduced. This is seen in Figure 4.24 & 4.25, which illustrate a reduction of the protein bands and also the band intensity. Coccoid form being the dormant form of *H. pylori* probably may not be as metabolically active as the spiral form. Nonetheless, this dormant form should be able to synthesize a minimum amount of proteins for its survival.

Several *H. pylori* proteins were identified after comparing the AGE preparations of the 2 forms (Table 4.24). Proteins identified include iron superoxide dismutase, 26 kDa protein, Non-heme iron containing ferritin (Pfr), neutrophil activating protein (NapA), thioredoxin and a putative protein. Results obtained from the database search showed that the putative protein is a conserved protein with no apparent known function.

The iron superoxide dismutase present in both spiral and coccoid form of *H. pylori* is a major defence mechanism against oxidative damage, catalyzes the breakdown of superoxide radicals to hydrogen peroxide and dioxygen. This enzyme acts to protect the pathogen from the lethal effects of toxic oxygen species.

The amino acid sequences of the 26 kDa protein shows similarities with alkyl hydroperoxide reductases (Ahp C) in both prokaryotes and eukaryotes (Storz *et al.*, 1989; Chae *et al.*, 1994). Alkyl hydroxide reductase belongs to the family of antioxidants known as the AhpC/TSA protein family. This group of enzymes reduce the harmful alkyl hydroperoxides to their corresponding alcohol and these enzymes are also produced by many bacterial species (Storz *et al.*, 1989). It has been shown that this protein is an antigen produced *in vivo* as patients infected with *H. pylori* can mount an immune response against this protein (Mattsson *et al.*, 1998). The essential of this protein is demonstrated by the study of Lunstrom & Bolin (2000) which attempted to construct a mutant deficient for the gene in *H. pylori* was unsuccessful. However, in a recent study by Olczak and colleagues (2002), *ahpC* mutant construct can be isolated if mutant selection is performed under low oxygen tension. They demonstrated that the mutants were more sensitive than the parent strain to oxidative stress-related chemicals. In the present comparative study, the 26kDa protein is observed in both forms. It is interesting to note a slight increase in the intensity of that specific protein band is seen in the coccoid form (Figure 4.24).

Two of the proteins which were detected with comparative quantity (similar band intensity) in both morphology forms are the non-heme iron containing ferritin (Pfr) and the neutrophil activating protein (NapA). The ferritin is a major iron storage protein of *H. pylori.* Waidner and colleagues (2002) have demonstrated that Pfr mediated iron storage is essential for survival of lethal iron starvation and protected the bacteria from acid amplified iron toxicity. They had also shown that the functions of *H. pylori* Pfr in iron metabolism were essential for the survival of *H. pylori* in the gastric mucosa, as the *pfr* mutant was unable to colonize in the Mongolian gerbils. This indicates that iron storage is

a prerequisite for the successful colonization of *H. pylori*. The protein identified as NapA, shared structure homology to the Dps family of proteins and Dps is implicated in the response of bacteria to oxidative stress (Tonello *et al.*, 1999). In addition, NapA has been shown to interact with DNA *in vivo* (Cooksley *et al.*, 2003). This interaction is postulated to protect against DNA damage. The low molecular weight protein identified as thioredoxin has also been shown to play important role in aiding the survival of the bacteria under oxidative stress (Comtois *et al.*, 2003).

Based on the proteins identified from current 1D-PAGE study, the coccoid form of *H. pylori* still exhibits proteins which probably play a part against the oxidative stress. Should the coccoid form be viable, these proteins may be undoubtedly important in its survival in the harsh aerobic environment outside the host.

5.6.2 Proteins identified from 2D-PAGE

The technique of 2D-PAGE is the heart of proteomic research. This technique separates proteins by molecular weight and *pI* of the proteins. In the present study, besides the 1D-PAGE, this methodology was employed to display and identified the proteins expressed by *H. pylori*. In accordance to that observed in 1D-PAGE (Figure 4.25), the number of proteins decreased at the various time course tested. Analysis of the proteome maps showed a ~45% reduction of proteins during morphological conversion, with 676 protein spots expressed in 3 day old culture to 374 spots expressed in 184 day old culture. The proteome maps show that majority of the proteins are presented near to the basic end (Figure 4.26). On the basis of the genome of *H. pylori*, about 70% of the encoded proteins have pI >7 (Tomb *et al.*, 1997).

In addition, the proteome profiles presented numerous proteins which appeared as trains of spots. Amongst the several *H. pylori* proteins which presented in multiple isoforms, two proteins were identified; Urease B and 26kDa (*tsaA*) (Figure 4.26). Lock *et al.* (2001) identified 12 protein spots for the gene coding *tsaA* and 5 proteins for *ureB*. The multiplicity of protein isoforms may be the consequence of post-translation modifications such as phosphorylation (Lock *et al.*, 2002). In another study by Lock *et al.* (2002), they showed that clusters of Urease B proteins were immunoreactive to both IgG and IgA. They also commented that though the importance of protein isoforms *in vivo* remains to be explored, the protein isoforms may have some bearing on the use of the recombinant versions of the *H. pylori* proteins as vaccine antigens.

The coccoid form of *H. pylori* maintains a level of the products of *tsaA* (Figure 4.27B). This protein is described earlier as having a role in the survival of *H. pylori* against oxidative stress environment. On the contrary, the Urease B is observed to be reduced significantly in the 30 day old culture. Nilsson *et al.* (2000) demonstrated a decline in Urease B in 12 day cell extracts as compared to an 8 day cell extracts. This observation is in agreement with that described by Zheng *et al.* (1999), the urease activity became weakly positive by day 28.

Several other proteins (Table 4.12) whose activities may maintain the viability of the bacteria are expressed in the aged culture. The results from the present differential study provide further evidence that the coccoid form is viable.

5.6.3 DIGE

DIGE is the use of pre-electrophoretic labelling with fluorescent dyes. The advantage of this method over the conventional 2D PAGE is the reduction of gel-to-gel variation as the samples are run together on the same gel. This methodology has been employed for normal proteomic analysis (Yan *et al.*, 2002) as well as for comparative differential study (Zhou *et al.*, 2001). However, the use of DIGE in the study of *H. pylori* has yet to be reported.

In the current study, we evaluate this new emerging technology by comparing the proteins expression in the spiral and coccoid *H. pylori* cells (Figure 4.29). The protein profiles are highly similar to that performed by the 2D-PAGE. One of the proteins was identified as the TagD protein (this protein was also identified in the silvered stained gel PAGE). From DIGE profile, 1 protein spot (adhesin-thiol peroxidase *tagD*) was up-regulated in the coccoid form. However, it is not clearly detected as up-regulated protein in coccoid cells by the 2D-PAGE. Hence, this methodology provided yet another feasible and useful proteomic technique which enables the differences between samples to be viewed within gel by using the specific software, DyCyder (Amersham Biosciences).

6.0 CONCLUSIONS

In the attempt to answer if the coccoid form of *H. pylori* plays a role in infection and transmission, the present study began with the familial clustering study. The results demonstrated clustering of *H. pylori* infection amongst family members using antibody profiling. The study presents the possibility of a vertical transmission. However, the mode of transmission remains elusive though it was postulated that the coccoid form which can

survive in extragastric environment may hold the key to the understanding of the transmission cycle. Thus, it leads one to postulate that children as the link in the transmission of *H. pylori* infection.

Earlier studies (Malaty *et al*., 2002; Perez-Perez *et al*., 2003) have pointed to the direction of *H. pylori* infection being acquired during childhood. In the current study, the immune responses of children having epigastric pain was evaluated using spiral as well as coccoid form as antigens in our in-house ELISA. The serological results showed a 2-fold increase in immune response against the spiral antigen in the symptomatic children as compared to the control group (asymptomatic school children of comparable age). Thus, inferring an association of *H. pylori* infection in children having epigastric pain. An interesting observation from the serology study is that a 4 fold increase in IgG level against the coccoid antigen in the children with epigastric pain was observed. This finding voiced a concerned question: Is there an under-reporting on the presence of *H. pylori* infection in this group of symptomatic young population?

The finding on the increase in immune response against the coccoid form of *H. pylori* in the symptomatic children and the many reports showing childhood as the period of acquisition of *H. pylori* infection, lead to the postulation that: could the infection have begun with the coccoid form. Thus, to be able to cause an infection, the coccoid forms would have to maintain its viability. Hence, we probed further by examining the coccoid form of *H. pylori* at the genomic and proteomics aspects.

The conversion of the spiral to coccoid form was observed over a time course. In this study, it was observed that the coccoid form exhibited fingerprinting profile highly similar to the spiral form. In addition, several putative determinants were conserved in the

coccoid form. The expression of the *26kDa* and *ureA* was also observed throughout the time course. Furthermore, the coccoid form was able to maintain the expression of the Le antigens as 2 peaks (at 5th & 30th day of culture) were noted in the time course study.

Proteomic study was carried out with 1D and 2D PAGE. Though the amount of proteins decreased during the morphological conversion, the protein profiles of the coccoid form was observed to be similar to the spiral form. Proteins from 1D and 2D PAGE identified by mass spectrometry showed that the coccoid form has retained the "essential" proteins (EF-TU, 26kDa, non-heme containing ferritin) as well as expressing proteins involved in combating oxidative stress. This will be an advantage if the coccoid form is to survive in the extragastric environment.

The innate response to the invading bacterial pathogen has shown a significant increasing IgG against the coccoid antigen. And yet, the coccoid form could not be resuscitated. Could it be that no suitable medium has been identified because no suitable culture medium has been identified as the knowledge or the understanding of the "inducer" that could awake or resuscitate the coccoid form to the spiral form in *in vitro* condition is not available. Nonetheless, this "inducer" could be readily available in *in vivo* condition.

Is the coccoid form a survival strategy that the bacterium adapts to survive in the extragastric environment? In the present study, comparative results from epidemiological and proteomics studies have lend support to the viability of the coccoid form of *H. pylori* and also suggesting that this form could play a key role in the transmission cycle of *H. pylori.*

Answering one or more of the questions regarding the coccoid form will help to shed light on the controversial point if the coccoid form is a degenerating form or VBNC

or viable entity of *H. pylori*. This will in turn provide impetus into the management of *H. pylori* infection and the probable role of coccoid form in transmission of *H. pylori* infection. One should therefore not overlook the close relationship of *H. pylori* infection in the paediatric patients with dyspeptic symptoms, in particular, epigastric pain.

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APPENDICES

APPENDIX 1

A1.1 Brain Heart Infusion (BHI) broth

For a 300 ml preparation,

Brain heart infusion (Gibco)	11.4 g
Yeast extract (Oxoid)	1.2 g
Distilled water	270 ml
Horse serum (10%) (Gibco)	30 ml

- Horse serum was added after the mixture of Brain heart infusion and yeast extract was autoclaved at 121°C for 15 minutes.

A1.2 Chocolate Blood agar (Non-selective)

For a 500 ml preparation,

Blood agar base No.2 (Oxoid)	20.0 g
Defibrinated horse blood (5%) (Gibco)	25 ml
Distilled water	475 ml

- Blood agar base No.2 was mixed with 475 ml distilled water.
- Mixture was autoclaved at 121°C for 15 minutes.
- The agar was cooled to 50°C before addition of 25 ml (5%) of horse blood.
- Mixture was swirled in a 80°C water bath for about 10 minutes to lyse the blood.
- Mixture was cooled before pouring into the sterile petri dishes.

A1.3 Chocolate Blood agar (selective)

Antibiotic Stock Solution

	Final concentration	Stock concentration
Vancomycin (Sigma)	3 µg/ml	3 mg/ml
Trimethoprim (Sigma)	5 µg/ml	5 mg/ml
Nalidixic acid (Sigma)	10 µg/ml	10 mg/ml
Amphotericin B (Sigma)	2 µg/ml	2 mg/ml

- Vancomycin and Nalidixic acid were prepared by dissolving the antibiotic powder in distilled water.
- Amphotericin B was dissolved in distilled water and adjusted to pH 11.0.
- Trimethoprim was dissolved in 70% ethanol and adjusted to the appropriate concentration with distilled water.
- The antibiotics were filtered using a sterile 0.22 µm filter and filtrate was aliquoted into eppendoff tubes.
- Aliquots were stored at -20°C until use.

Chocolate blood agar supplemented with antibiotics

For a 500 ml preparation,

Chocolate blood agar (Section A1.2)	489 ml
Vancomycin stock solution	0.5 ml (Final concentration: 3 µg/ml)
Trimethoprim stock solution	0.5 ml (Final concentration: 5 µg/ml)

Nalidixic acid stock solution	0.5 ml (Final concentration: 10 μg/ml)
Amphotericin B stock solution	0.5ml (Final concentration: 2 μg/ml)

- Chocolate blood agar was prepared as described in Section A1.2 and cooled to 50 ^{o}C.
- The antibiotic stock solutions were added to the cooled molten chocolate blood agar.
- The well-mixed chocolate blood agar with antibiotics was poured into sterile petri dishes.
- The agar plates were stored at 4°C until use.

APPENDIX 2

A2.1 Urease Test Reagents

For 50 ml preparation,

Urea (Sigma)	1g
Phenol red (0.5% w/v)	5 ml
$Na_2H_2PO_4 . H_2O$	21.8 µg
Na_2HPO_4	51 µg

- Urea and the inorganic salts were dissolved in 45 ml of distilled water.
- The pH was adjusted to pH 6 before use.
- Mixture was autoclaved at 121°C for 10 minutes.
- Filtered phenol red indicator was then added.

APPENDIX 3

Buffers and Reagents for ELISA

A3.1 0.05 M Carbonate Buffer (pH 9.6)

Na_2CO_3	1.59 g
$NaHCO_3$	2.93 g
Distilled water (qsp)	1L

A3.2 10x Phosphate buffered saline (PBS), pH 7.4

For 1L preparation,

NaCl	80 g
Na_2HPO_4	14.4 g
KCl	2.8 g
KH_2PO_4	2.4 g
Distilled water (qsp)	1L

A3.3 Serum Diluent

PBS (1x)	1L
Tween 20 (Merck)	0.5 ml
Thimerosal (Sigma)	0.2 g
Gelatin (Gibco)	1 g (dissolve by heating)

A3.4 Wash Buffer I

PBS (1x)	1L
Tween 20	0.5 ml
Thimerosal	0.2 g

A3.5 Conjugate Diluent

PBS (1x)	1 L
Thimerosal	0.2 g
Gelatin	1g (dissolved by heating)
Bovine serum albumin (BSA)(Sigma)	0.02 g

A3.6 Wash Buffer II

PBS (1x)	1L
Thimerosal	0.2 g

A3.7 Phosphate Citrate Buffer (Substrate Buffer), pH 5

For 500 ml preparation,

0.1 M Citric acid (Merck)	2.55 g
0.2 M $NaHPO_4.2H_2O$	4.57 g
Distilled water (qsp)	500 ml

APPENDIX 4

Buffers and Reagents for polyacrylamide gel electrophoresis

A4.1 2X Treatment Buffer

4% SDS	4 ml from 10% SDS
0.5 M Tris-HCl (pH 6.8)	2.5 ml
ß-mecaptoethanol (BDH)	1.0 ml
Glycerol (Merck)	2.0 ml
Bromophenol Blue (Bio-Rad)	0.005 g
Distilled water	0.5 ml

A4.2 Resolving gel Buffer (pH 8.8)

For 500 ml preparation,

1.5 M Tris	90.75 g
Distilled water (qsp)	500 ml

Adjust to pH 8.8 with HCl

A4.3 Stacking gel Buffer (pH 6.8)

For 500 ml preparation,

0.5 M Tris	30.17 g
Distilled water (qsp)	500 ml

Adjust to pH 6.8 with HCl

A4.4 10% SDS

SDS (Sigma)	10 g
Distilled water (qsp)	100 ml

A4.5 10% Ammonium persulphate (APS)

APS (Bio-Rad)	1 g
Distilled water (qsp)	10 ml

A4.6 10x Tank Buffer

Tris	30.28 g
Glycine (BDH)	144.13 g
SDS	10 g
Distilled water (qsp)	1L

A4.7 Overlay Solution

Isopropanol or water saturated n-butanol

A4.8 Resolving gel and stacking gel recipes

	4%	12%
Acrylamide-bis solution (30%) (Bio-Rad)	3.3 ml	40 ml
Resolving gel buffer	-	25 ml
Stacking gel buffer	6.3 ml	-
10% SDS	250 μl	1.0 ml
10% APS	125 μl	500 μl
Temed (Bio-Rad)	25 μl	50 μl
Distilled water	15 ml	33.45 ml
Total Vol	25 ml	100 ml

APPENDIX 5

Buffers and Reagents for western blotting (Semi-dry electrophoretic transfer)

A5.1 Transfer Buffer (pH 9.2)

48 mM Tris	5.82 g
39 mM glycine	2.93 g
1.3 mM SDS	3.75 ml from 10% SDS
20% Methanol	200 ml
Distilled water (qsp)	1L

A5.2 Blocking Solution

Skim milk powder	5 g
PBS Tween-20	100 ml

A5.3 Wash Buffer (PBS-Tween-20)

For 1L preparation,

PBS	1L
Tween 20	0.5 ml

A5.4 Substrate Solution

4-chloro-1-napthol (Sigma)	60 mg
Cold methanol	20 ml
PBS	100 ml

- Substrate, 4-chloro-1-napthol was dissolved in methanol in the dark.
- 100 ml of PBS was added to the substrate and 60 µl of 30% hydrogen peroxide was added immediately before use.

APPENDIX 6

Buffers and Reagents for DNA extraction and PCR

DNA extraction

A6.1 TE Buffer

1 M Tris (pH 8)	1 ml	(Final conc: 10 mM)
0.5 M EDTA (pH 8)(Sigma)	0.2 ml	(Final conc: 1mM)
Distilled water	98.8 ml	

PCR reaction

A6.2 50X TAE BUFFER

Tris	242 g
Glacial acetic acid	57.1 ml
EDTA	18.6 g
Distilled water (qsp)	1L

- Buffer was diluted to 1X before use.

A6.3 Loading Buffer (5X)

Glycerol	3 ml
Bromophenol Blue	0.025 g
Xylene Cyanol	0.025 g
Distilled water (qsp)	10 ml

APPENDIX 7

Buffers and Reagents for 2D PAGE

A7.1 Lysis Buffer

9 M Urea	9.8 g
4% CHAPS (UBS)	0.8 g
40 mM Tris	533 µl
Complete, Mini protease inhibitor Cocktail tablets (Roche)	1 tablet
Distilled water (qsp)	20 ml

A7.2 Rehydration stock solution with IPG Buffer

8 M Urea	12 g
2% CHAPS (w/v)	0.5 g
0.5% IPG Buffer	125 µl
0.001% Bromophenol blue (Bio-Rad)	0.25 mg or a few grains
Distilled water (qsp)	25 ml

- Aliquots of 1ml amount were kept at -20ºC.
- 15 mg dithiothreitol (DTT) per 1ml aliquot was added prior to use.

A7.3 SDS Equilibration Buffer

50 mM Tris	6.7 ml from 1.5 M Tris (pH 8.8)
6 M Urea	72.07 g
30% Glycerol (87% v/v)	69 ml
2% SDS	4 g
0.001% Bromophenol blue (Bio-Rad)	0.002 g
Distilled water (qsp)	200 ml

- Aliquots were stored in 10 ml amount at -20ºC until use.
- 100 mg DTT or 250 mg Iodoacetamide were added to the 10 ml buffer prior to use.

APPENDIX 8

2D DIGE preparation

A8.1. Cell wash buffer

10 mM Tris (pH 8)	12 mg
Distilled water (qsp)	10 ml

A8.2 Lysis Buffer

30 mM Tris	36 mg
7 M Urea	4.2 g
2 M Thiourea (Sigma)	1.52 g
4% CHAPS (USB)	0.4 g
Distilled water (qsp)	10 ml

- pH was adjusted to pH 8 with diluted HCl. Aliquots of 1ml amount were stored at -20ºC until use.

A8.3 2X Sample Buffer

8 M Urea	4.8 g
130 mM DTT (Bio-rad)	0.2 g
4% Chaps	0.4 g
2% (v/v) Pharmalyte TM 3-10	0.2 ml
Distilled water (qsp)	10 ml

- Aliquots of 1ml amount were stored at -20ºC until use.