SPECIFIC *HELICOBACTER PYLORI* VIRULENCE AND HOST GENETIC SUSCEPTIBILITY FACTORS: THE POTENTIAL ROLE IN GASTRODUODENAL DISEASES

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AGCC	Affymetrix genechip command console software
AlpAB	Adherence–Associated Lipoprotein
ARHGAP26	Rho GTPase activating protein 26
ATCC	American Type Culture Collection
AW1	Wash buffer 1
AW 2	Wash buffer 2
BabA	Blood Group Antigen Adhesin
BANK1	B-cell scaffold protein with ankyrin repeats 1
BHI	Brain Heart Infusion
BLAST	Basic Local Alignment Search Tool
bp	Basepair
BSA	Bovine serum albumin
^{o}C	Celsius gradient
CagA	Cytotoxin-Associated Gene A protein
*cag*PAI	*cag* Pathogenicity Island
CD-CV	Common disease-common variant
CDH13	cadherin 13
CM	CagA Multimerization
CNV	Copy number variation
CO2	Carbon dioxide
13C	Carbon-13
14C	Carbon-14
Csk	Carboxy-Terminal Src Kinase
DDBJ	DNA Database of Japan
DNA	Deoxyribose nucleic acid
dNTPs	Deoxynucleotid Triphosphate
DRAM1	DNA-damage regulated autophagy modulator 1
DSB	Double strand breaks
dsDNA	Double strand DNA
DU	Duodenal Ulcer
dupA	Duodenal Ulcer Promoting Gene A
E-CM	East Asian- CagA Multimerization
EDTA	Ethylene Diamine Tetra Acetic Acid
EMBL	European Molecular Biology Laboratory
EPIYA motif	Glu-Pro-Ile-Tyr-Ala Motif
EtOH	Ethanol
GERD	Gastro-esophageal reflux disease
GC	Gastric Cancer
GTC	Genotype console software
GU	Gastric Ulcer
HWE	Hardy Weinberg Equilibrium
GWAS	Genome wide association study
H. pylori	*Helicobacter pylori*
HopZ	*H. pylori* Outer Membrane Protein Z
IARC	International Agency for Research in Cancer
iceA	Induced by Contact to Epithelium
MAF	Minor allele frequency

MALToma	Mucosa Associated Lymphoid Tissue Lymphoma
MAST4	microtubule associated serine/threonine kinase family member 4
min	Minute
mg	miligram
ml	Mililitre
µl	Microliter
µg	Microgram
µM	Micromole
MLST	Multi Locus Sequence Typing
MMEJ	Microhomology-mediated end joining
MMR	Mismatch Repair System
MPCR	Multiplex PCR
n	Number
RUT	Rapid Urease Test

KEVIRULENAN KHUSUS *HELICOBACTER PYLORI* DAN FAKTOR KERENTANAN GENETIK PERUMAH: POTENSI PERANAN TERHADAP PENYAKIT GASTRODUODENAL

ABSTRAK

Helicobacter pylori (H. pylori) adalah salah satu patogen manusia yang paling lazim dan memberi kesan terhadap 50% daripada populasi manusia. *H. pylori* dikaitkan dengan penyakit gastrik, ulser peptik, kanser gastrik dan limfoma berkaitan dengan tisu limfoid mukosa gastrik. Tindak balas beberapa faktor seperti persekitaran, kevirulenan bakteria dan genetik hos dipercayai boleh menentukan tahap kemudaratan dan kesan selepas jangkitan *H. pylori*. Tujuan kajian ini adalah untuk menentukan distribusi gen-gen virulen *H. pylori* (*cagA, babA2*, *SabA* dan *dupA*) dan korelasinya dengan hasil klinikal. Kajian ini juga bertujuan menilai corak *H. pylori cagA* motif EPIYA; EPIYA-A, -B, -C atau -D di antara kumpulan etnik dan perkaitannya dengan penyakit gastroduodenal. Kajian ini turut mengenalpasti kehadiran SNP sebagai varian genetik di dalam genom perumah yang mungkin berkait dengan kerentanan atau pertahanan tarhadap jangkitan *H. pylori*. Ini adalah kajian keratan rentas dan kajian kes kawalan di antara Mei 2012 sehingga Jun 2014 dalam kalangan pesakit dispeptik dan berlainan kaum (Melayu, India dan Cina) di Unit Endoskopi Hospital Universiti Sains Malaysia dan Hospital Kuala Lumpur. Genotyping genom perumah dilakukan dengan mengunakan teknik PCR dan Affymetrix SNP microarray 6.0. Kajian ini merangkumi dua fasa; dalam fasa pertama, sejumlah 105 pesakit yang disahkan positif terhadap jangkitan *H. pylori* telah terlibat dalam kajian ini. Purata umur dan SD adalah 54.48+12.94 tahun dengan julat umur di antara 26 sehingga 86 tahun. Lima puluh tujuh (54.3%) pesakit yang dijangkiti adalah lelaki manakala empat puluh lapan (45.7%) adalah wanita. Berdasarkan penemuan endoskopik, 78 pesakit mengalami gastritis, sembilan gastrik ulser, lima ulser duodenal dan 13 normal. Penemuan gen-gen *H. pylori cagA, babA2*, *sabA*2 dan *dupA* dalam pesakit dispeptik *H. pylori* masing-masing adalah 69.5%, 41.0%, 43.8% dan 22.9%. Gen *cagA* dikesan dengan lebih tinggi dalam kalangan bangsa India (39.7%), *babA2* lebih lazim bagi Melayu (39.5%) manakala *dupA* adalah tertinggi bagi kalangan bangsa India dan Melayu dengan kadar yang sama (37.5%). Bangsa Cina mempunyai kelaziman paling rendah terhadap keempat-

empat gen. Majoriti pesakit Cina dijangkiti dengan *cagA* jenis A-B-D strain Asia Timur (88.9%) manakala *cagA* jenis A-B-C strain Barat (82.8%) dikesan lebih tinggi dalam kalangan bangsa India. Bangsa Melayu mempunyai strain bercampur. Terdapat perkaitan yang signifikan secara statistik (**p<0.001**) di antara etnik dan *cagA* motif EPIYA, walaupun tiada perbezaan signifikan di antara gen virulen *H. pylori* dan jenis EPIYA dengan hasil klinikal. Dalam fasa kedua, sejumlah 80 (42 *H. pylori* positif) dan 38 (*H. pylori* negatif) pesakit generasi ketiga dengan purata umur 49.87+12.335 (umur di antara 20-75 tahun). Kajian ini menunjukkan bahawa SNP rs3770521 (**P=1.33 X10^{-5}**) gen XRCC5, rs7042986 (**P=0.0001**) gen SMARCA2, dan rs10860808 (**P=0.0002**) gen DRAM1 adalah SNP yang cenderung kepada jangkitan *H. pylori* dalam kalangan pesakit gastrik berbangsa India, Melayu dan Cina. Kajian ini turut mengenalpasti dua SNP yang protektif, iaitu rs1809758 (**P=9.85X 10^{-6}**) gen BANK1 dan rs3776349 (**P=0.0001**) gen ARHGAP26 dalam kalangan pesakit gastrik India dan Melayu. Kesimpulannya, gen yang rendah dan variasi dalam kumpulan etnik yang berlainan menunjukkan strain bakteria tersebut bergantung kepada perbezaan etnik dan geografi. Kajian ini juga menunjukkan bahawa tiada perbezaan yang signifikan di antara gen virulen dengan hasil klinikal. Kajian ini turut membuktikan bahawa EPIYA A-B-D dan A-B-C adalah predominan dalam bangsa Cina dan India manakala bangsa Melayu mempunyai strain campuran. Akhir sekali, kajian GWAS terkini menunjukkan lima SNP unggul yang boleh dikaitkan dengan kecenderungan dan ketahanan terhadap *H. pylori* gastritis dalam ketiga-tiga kumpulan etnik ini.

SPECIFIC *HELICOBACTER PYLORI* VIRULENCE AND HOST GENETIC SUSCEPTIBILITY FACTORS: THE POTENTIAL ROLE IN GASTRODUODENAL DISEASES

ABSTRACT

Helicobacter pylori (H. pylori) is one of the most common human pathogens and affects over 50% of the world population. *H. pylori* is associated with gastritis, peptic ulcer, gastric cancer and gastric mucosa-associated lymphoid tissue lymphoma. The interaction of several factors like environmental, bacterial virulence and host genetic are believed to determine the severity and final outcome after *H. pylori* infection. The aim of this study was to determine the distribution of *H. pylori* virulence genes (*cagA, babA2, SabA*and *dupA*) and its correlation with clinical outcomes. This study also assessed the pattern of *H. pylori cagA* EPIYA motifs, EPIYA-A, -B, -C, or -D among different ethnic groups and its association with gastroduodenal disease. The current study also explored the presence of SNPs as genetic variants in the host genome which may be associated with susceptibility or protection to *H. pylori* infection. This was a cross-sectional and case-control study conducted between May 2012 to June 2014 among dyspeptic patients of different ethnicities (Malay, Indian and Chinese) at the Endoscopy Unit of Hospital Universiti Sains Malaysia and Hospital Kuala Lumpur. Genotyping of bacterial and host genome was performed using PCR and Affymetrix SNP 6.0 microarray. This study consists of 2 phases; in phase 1, a total of 105 patients who were confirmed positive to have *H. pylori* infection were recruited into the study. The mean age and SD were 54.48 ±12.94 years and age range of 26 to 86 years old. Fifty seven (54.3%) of the infected patients were males while forty eight (45.7%) were females. Based on the endoscopic findings, 78 patients had gastritis, nine gastric ulcer, five duodenal ulcer and 13 normal. The prevalence of *H. pylori cagA, babA2, sabA* and *dupA* genes in *H. pylori* dyspeptic patients were 69.5%, 41.0%, 43.8% and 22.9% respectively. *cagA* is more common in Indians (39.7%), *babA2* is common in Malays (39.5%) and *dupA* detection is more in Indian and Malay at the same rate (37.5%). The Chinese have

the lowest prevalence of the four genes. Majority of Chinese patients were predominantly infected with *cagA* type A-B-D East Asian strain (88.9%) while *cagA* type A-B-C Western strain (82.8%) was predominantly detected in the Indians while the Malays have mixed strain. There were statistically significant difference (***P*<0.001**) between ethnicity and *cagA* EPIYA motifs, although we could not find significant difference between *H. pylori* virulence genes and EPIYA types and clinical outcomes. In phase II, a total of 80 (42 *H. pylori* positive and 38 *H. pylori* negative) third generation patients with a mean age of 49.87 ± 12.335 years (age range 20-75 years) were recruited. The present study identified SNPs rs3770521 (***P*=1.33 x 10^{-5}**) of XRCC5 gene, rs7042986 of SMARCA2 (***P*=0.0001**) and rs10860808 (***P*=0.0002**) of DRAM1 gene as the susceptible SNPs to *H. pylori* infection among the Indian, Malay and Chinese gastritis patients respectively. This study also identified two protective SNPs rs1809578 (***P*=9.85x 10^{-6}**) of gene BANK1 and rs3776349 (***P*=0.0001**) of gene ARHGAP26 among *H. pylori* the Indian and Malay gastritis patients respectively. In conclusion, the lower prevalence of virulence genes and variations among the different ethnic groups suggest that the bacterial strains are geographically and ethnically dependent. No significant difference was observed between virulence genes and clinical outcome. This study also shows that EPIYA A-B-D and A-B-C are predominant in the Chinese and Indians respectively, while the Malays have mixed strain. Finally, the current GWAS study revealed five novel SNPs that may be associated with susceptibility and protection of *H. pylori* gastritis in the three ethnic groups.

Chapter 1: Introduction

1.1 *H. pylori*

Marshall and Warren, in 1982 were the first to isolate and culture the spiral bacterium, now known as *H. pylori*, from the gastric mucosa of humans (Marshall and Warren, 1984). In their first study of more than 100 antral biopsy samples, they observed this bacterium to be present by histology in 58 subjects undergoing endoscopic examination and were able to isolate and culture it from 11 biopsy specimens. Based on these results, Marshall and Warren suggested that there was an etiological relationship between *H. pylori* and these gastric diseases (Marshall and Warren, 1984). Therefore in 2005 as recognition of the value of their discovery to the medical world, Barry Marshall and Robin Warren were awarded the Nobel Prize in Medicine.

1.2 Historical aspects

Prior to the discovery by Marshall and Warren, other scientists had also observed this bacteria to be present in the gastric mucosa of both humans and animals. The first of such report came from Bottcher in 1874 in which he described the bacteria to be present on the ulcer floor and in the mucosal margins of ulcers. In 1938, Doenges found spiral organisms in 103 (43%) of 242 stomachs examined at autopsy. In 1954, however, Palmer reported no evidence of spiral organisms in 1180 gastric mucosal biopsies from 1000 patients; he suggested that the organism noted previously in normal stomachs represented postmortem processes and that the

bacterial source was the oral cavity. In 1975, Steer and Colin-Jones reported the presence of Gram-negative bacteria in the gastric mucosa of approximately 80% of patients with gastric ulcers (Ha, 2007).

Due to the low pH of gastric acid, the human stomach was long believed to be a sterile environment. Although some isolated reports had been made regarding the existence of bacteria in the stomach, since the 1800's, it was not until 1982 that the major breakthrough in the understanding of gastric and duodenal pathology such as gastritis, peptic ulcer and gastric cancer occurred when Robin Warren and Barry Marshall described "unidentified curved bacilli on gastric epithelium in active chronic gastritis (Robin Warren and Marshall, 1983).

1.3 Taxonomy

H. pylori was originally considered as a Campylobacter-like organism (CLO) and was named *Campylobacter pyloridis* (Marshall and Warren, 1984), based on bacterial similarity with other Campylobacter species. In 1987 this name was corrected for grammatical reasons to *Campylobacter pylori* (Marshall and Goodwin, 1987). However, based on differences in the 16S rRNA gene sequences, fatty acid profiles and flagella morphology, this bacterium was placed in new genus called *Helicobacter* with the new name for this bacterium being *H. pylori*. The name "*Helicobacter*" was based on the helical shape of the bacterium and the word "pylori" was used because the bacterium was commonly isolated from the pylorus of the stomach (Goodwin *et al.*, 1989).

1.4 Microbiological features and growth requirements

Helicobacter belongs to the family Helicobacteraceae, order Campylobacterales and the Epsilonproteobacteria class (Owen, 1998). *H. pylori* is a Gram negative spiral rod with 2 – 4 μm in length and 0.5 – 1μm wide. The bacterium can appear as rod, while coccodi shapes appear after prolonged in *vitro* culture or antibiotic treatment (Kusters *et al.*, 1997). It has 2 to 6 unipolar, sheathed flagella of nearly 3 μm in lengths, which frequently carry a unique bulb at the end. The main function of flagella is to allow quick movement in viscous solutions such as the mucus layer overlying the gastric epithelial cells (O'Toole *et al.*, 2000).

H. pylori is a slow-growing microaerophiles, fastidious microorganism and require complex growth media. It grows optimally at 37°C on a rich medium containing blood or serum. These supplements may act as additional sources of nutrients and possibly also protect against the toxic effects. Media mostly used for routine isolation and culture of *H. pylori* consist of Columbia or Brucella agar supplemented with either (lysed) horse or sheep blood or, alternatively, new-born or fetal calf serum. For isolation, selective antibiotic mixtures are available (Owen, 1998; Ndip *et al.*, 2003). Dent supplement consists of vancomycin, trimethoprim, cefsoludin, and amphotericin B, whereas Skirrow supplement consists of vancomycin, trimethoprim, polymyxin B, and amphotericin B. Liquid media usually consist of Brucella, Mueller-Hinton, or brain heart infusion broth supplemented with 2 to 10% calf serum or 0.2 to 1.0% -cyclodextrins, often together with either Dent or Skirrow's supplement (Ndip *et al.*, 2003). *H. pylori* can be identified by Gram staining, oxidase, catalase, and urease test (Maaroos *et al.*, 2004).

Currently, at least 32 *Helicobacter* species have been identified in humans and animals (http://www.bacterio.cict.fr/h/helicobacter.html). These are generally divided into two groups based on the niche they colonise: 'gastric' Helicobacters primarily colonise the stomach whilst 'enterohepatic' Helicobacters colonise the intestine and hepatobiliary system.

1.5 Epidemiology

1.5.1 Prevalence

H. pylori infects more than half of the world's population (Ryan *et al.*, 2001; Amjad *et al.*, 2010). The prevalence was around 25% in developed countries to more than 90% in developing areas, but not all infected individuals eventually developed the disease (Figure 1.1) (van Doorn *et al.*, 2000; Ribeiro *et al.*, 2003). The prevalence of *H. pylori* infection varies widely by geographic area, age, race, and socioeconomic status (Brown, 2000). The prevalence varies from one country to another for example in Australia, it is as low as 15.5% (Pandeya and Whiteman, 2011), while in India, as high as 87% (Miwa *et al.*, 2002). Among East Asian countries, the overall seroprevalence rate was 59.6% in South Korea (Yim *et al.*, 2007), 58.07% in China (Wang and Wang, 2003), 54.5% in Taiwan and 39.3% in Japan (Fock, 2014). Among Southeast Asian countries, the reported sero prevalence rate was 35.9% in Malaysia (Goh and Parasakthi, 2001), 31% in Singapore (Fock, 2014) and 57% in Thailand (Deankanob *et al.*, 2006).

H. pylori prevalence varies considerably between the three primary ethnic groups resident in Malaysia, Malays, Chinese and Indians with prevalence rates of 8-43.3%, 45-60.6% and 68.9-75% respectively (Ramelah *et al.*, 2005; Tan *et al.*, 2005). *H. pylori* acquisition is declining in developed countries at a faster rate as compared to developing countries, because of the improvement in hygiene practices in the developed world (Brown, 2000). In 1994, on the basis of various epidemiological studies, *H. pylori* was classified as a class I carcinogen in humans by a working group of the World Health Organization International Agency for Research on Cancer (Yamazaki *et al.*, 2005b).

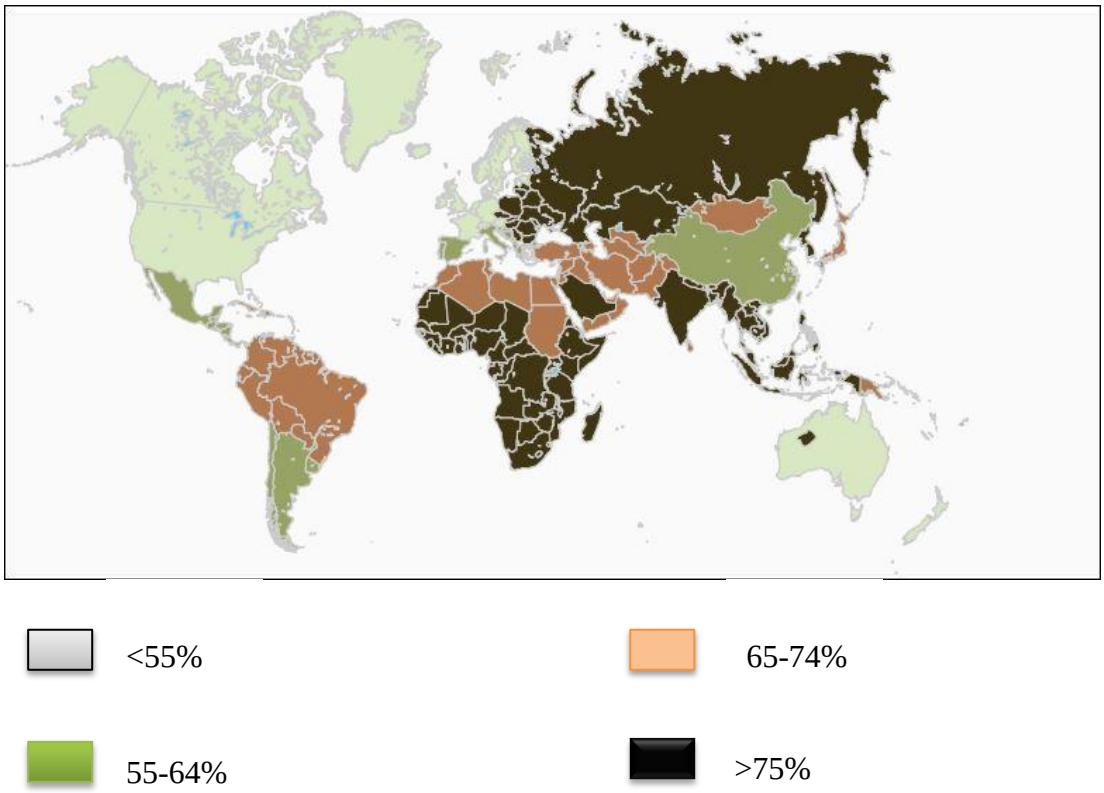

Figure 1.1 World-wide distribution of *H. pylori* infection adapted from (Bauer and Meyer, 2011)

1.5.2 Transmission and source of infection

The exact mechanisms whereby *H. pylori* is acquired are largely unknown. *H. pylori* is almost always acquired in childhood. The only known reservoir for *H. pylori* is the human stomach. Person to person spread appears to be the most likely mode of transmission (Quinn *et al.*, 2003) Transmission has been documented by vomitus, saliva, or faeces and possibly also through water source in the developing world (Brown *et al.*, 2002).

Convincing evidence of intrafamilial transmission, particularly parent to child, has been provided by various studies showing an intrafamilial clustering of infection (Kivi *et al.*, 2003; Perez-Perez *et al.*, 2004; Schwarz *et al.*, 2008; Nahar *et al.*, 2009). Studies have found that iatrogenic transmission occurs through use of a variety of inadequately disinfected gastric devices, endoscopes, and endoscopic accessories. Adequate sterilization and disinfection of endoscopes has been found to reduce the incidence of transmission (Muhammad *et al.*, 2012).

Faeco-oral transmission and oral-oral transmission of bacteria have been reported. Contaminated water supplies in developing countries may serve as an environmental source of bacteria. Children who regularly swim in rivers, streams, pools, drink stream water, or eat uncooked vegetables have greater chance of infection (Muhammad *et al.*, 2012). In the absence of treatment, infection is usually lifelong. The major risk factor for infection is poor socio-economic conditions in childhood (Malaty and Graham, 1994), overcrowding and ethnic and genetic predisposition (Das and Paul, 2007).

1.5.3 *H. pylori* as a tool for tracking human migration

H. pylori strains from different geographic areas demonstrate phylogeographical differentiation; therefore, the genotypes of *H. pylori* strains can serve as markers of the migration of human populations. Thus, the genotypes of *H. pylori* virulence factors, *cagA* and *vacA*, as well as multilocus sequence typing (MLST), are widely used markers of genomic diversity in *H. pylori* populations. The *cagA* virulence factor has two types: the East Asian and the Western types (Yamaoka *et al.*, 2000b; Yamaoka, 2009). *H. pylori* infection has rapidly declined because personal hygiene and quality of life have improved. The molecular epidemiology of *H. pylori* infection is highly informative and should be investigated before this characteristic is completely lost.

The MLST of seven housekeeping genes has been obtained from several *H. pylori* strains isolated from different geographical and ethnic origins; MLST results have shown that *H. pylori* has followed human migration from Africa (Falush *et al.*, 2003). For instance, six *H. pylori* populations, including hpAfrica1, hpAfrica2, hpNEAfrica, hpEurope, hpEastAsia, and hpAsia2, have been identified, indicating *H. pylori* has migrated with its host from Africa (Falush *et al.*, 2003). hpEastAsia comprises hspAmerInd, hspMaori, and hspAsia; hpEurope includes strains from Europe, Turkey, Bangladesh, Ladakha (India), Sudan, and Israel. Interestingly, isolates from Europe form a heterogeneous population, in which the modern hpEurope population is a combination of two ancestral European populations that likely settled in Europe in different waves (Falush *et al.*, 2003). A recent study done in Malaysian Malay, Chinese and Indian showed variations in population. hpEastAsia has been isolated from Chinese patients; furthermore, hpAsia2 isolates

have been obtained from Indians. Isolates from Malay patients comprise a mixed group; these isolates are similar to hspIndia subpopulation (Falush *et al.*, 2003; Tay *et al.*, 2009). The low prevalence of infection and the variation of Malay isolates imply that Malays were originally free of *H. pylori* but recently acquired the pathogen from other subpopulations, mainly from Indians (Tay *et al.*, 2009). It has been established that *H. pylori cagA* EPIYA patterns have a significant geographic variability and closely follow patterns of historical human migrations. EPIYA D is a characteristic Asian EPIYA pattern that virtually does not occur in the Western *H. pylori* strains (EPIYA C) (Queiroz *et al.*, 2010).

1.6 Pathogenicity and virulence factors of *H. pylori*

H. pylori is a Gram-negative spiral bacterium that colonizes and persists in human gastric mucosa. *H. pylori* infects more than half of the world's population, and it is implicated as a causative agent of gastritis, peptic ulcer disease, carcinoma, and mucosa-associated lymphoid tissue lymphoma (Ryan *et al.*, 2001; Bindayna *et al.*, 2006). It is one of the most common bacterial infections in humans (Blaser, 1997). *H. pylori* strains have been divided into two broad families, type I and type II, which are based on whether or not they possess the *vacA* and *cagA* genes. Type I strains have the ability to produce *VacA* and *CagA*, while type II strains lack that ability. Type I strains are regarded as having greater pathogenicity and potential to cause development of disease (Yamazaki *et al.*, 2005b). *H. pylori* possesses a number of virulence factors that allow it to colonize the harsh environment of the stomach, with many of these factors also implicated in its pathogenesis. Among these are urease, flagella, *cagA*, *cagA* EPIYA motifs, *vacA,dupA, babA and sabA.*

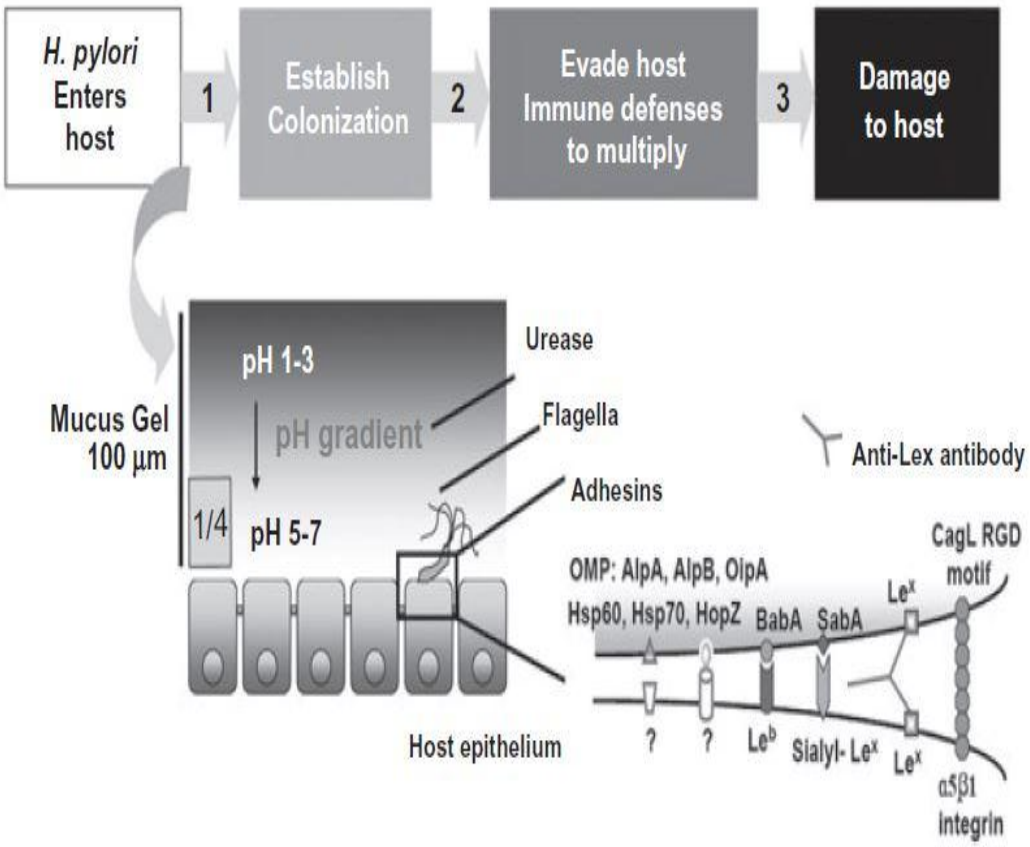

Figure 1.2 Schematic diagram of colonization as the first step in *H. pylori* infection adapted from (Sheu *et al.*, 2010).

Figure 1.2 shows the urease activity and flagella of *H. pylori* facilitate its orientation to the lower one-fourth of the mucus gel above the epithelium. Several putative adhesion molecules, including *babA*, *SabA*, Lewis antigen, and other outer membrane proteins (OMPs) are ready to adhere to known or unknown counterpart receptors on the host the epithelium. (Sheu *et al.*, 2010).

1.6.1 Urease

One of the prominent features of *H. pylori* its ability to colonize the acidic gastric environment, although the bacterium is not an acidophile it overcomes the acidic conditions by production of urease enzyme (Kusters *et al.*, 2006). *H. pylori* survives at a pH range between 4.0 and 8.0 in the absence of urea. However, in the presence of urea the organism can survive at a pH as low as 2.5 (Dunne *et al.*, 2014).

In the stomach the *H. pylori* urease enzyme converts urea into ammonia and carbon dioxide. The ammonia helps to neutralize the acidic environment around the bacterium and enable *H. pylori* to survive and multiply in the stomach (Andersen, 2007).

1.6.2 Flagella

Motility is essential for *H. pylori* colonization. Flagellar motility is thought to be required for the initial stages of infection allowing the bacterium to move from acidic environment of the gastric lumen into less acidic mucus layer (Thompson *et al.*, 2003). *H. pylori* carries 5-7 sheathed flagella that perform the motility required for colonization and infection. The flagella filament consists of two subunits. Both genes coding for these flagellins are necessary for full motility of *H. pylori*. The sheathing of the flagella is believed to protect them from the acidic environment in the stomach.(Andersen, 2007).

1.6.3 Cytotoxin-associated gene A (*cagA*)

CagA is the most widely studied *H. pylori* virulence factor and is present in many but not all *H. pylori* strain (Covacci *et al.,* 1993). The *cagA* gene is located at one end of the cag pathogenicity island (PAI) that codes a type IV secretion system (T4SS) linked with increased secretion of IL-8, a very strong pro-inflammatory chemokine that participates in the gastritis induced by *H. pylori* infection. The T4SS is also liable for the entrance of *cagA* protein into the gastric epithelial cells. After the delivery, *cagA* protein is quickly tyrosine phosphorylated on specific tyrosine residues within repeating Glu-Pro-Ile-Tyr-Ala (EPIYA) motifs and interacts with various target molecules (Naito *et al.,* 2006). Studies have shown that knockout mutants of TFSS genes could not undertake *cagA* transfer and as a result *cagA* tyrosine phosphorylation did not occur. This means that for a functional *cagA* translocation, the TFSS genes must be intact (Stein *et al.,* 2000).

The phosphorylated EPIYA motifs of *cagA* interact and activate the Src homology 2 (SH2) domain-containing tyrosine phosphatase (SHP-2) of the host cells. The *cagA*-SHP-2 complex leads to change in proliferation, morphogenesis and motility of gastric epithelial cells and hence inducing the "hummingbird" phenotype (cell elongation). As SHP-2 plays an important role in both cell growth and cell motility, deregulation of SHP-2 by *cagA* may be involved in the induction of abnormal proliferation and movement of gastric epithelial cells, a cellular condition eventually leading to gastritis and gastric carcinoma (Backert *et al.,* 2001; Higashi *et al.,* 2002a; Yamazaki *et al.,* 2003).

CagA-positive *H. pylori* strains are most virulent strains and are associated with higher risk for peptic ulcers (Oleastro *et al.*, 2003; Ribeiro *et al.*, 2003; Yamazaki *et al.*, 2005b). Several studies based on a few strains demonstrated that *cagA*-positive *H. pylori* isolates, but not *cagA*-negative isolates, were able to induce interleukin 8 (IL-8) secretion in *vitro* and in *vivo* (Huang *et al.*, 1995; Sharma *et al.*, 1995). Therefore the ability to induce IL-8 secretion is recognized as one of the major virulence factors of *H. pylori* and seems to be important in the establishment of PUD (Peek *et al.*, 1995).

It has been reported that *cagA* gene is present in approximately 60% of *H. pylori* strains from Western populations, but in contrast, it is present in over 90% of the strains from Southeast Asian populations (Chen *et al.*, 2005; Siavoshi *et al.*, 2005). *CagA* gene is considered as a marker to predict the severity of peptic ulcer disease in European and North American populations (Covacci *et al.*, 1993). The presence of *cagA* has been associated with peptic ulcer disease and gastric cancer (Miehlke *et al.*, 2001).

1.6.3.1 *cagA* EPIYA motifs

cagA is a polymorphic gene that presents different numbers of repeated sequences located in its 3' region. Each repeated region of *cagA* protein contains Glu-Pro-Ile-Tyr-Ala (EPIYA) motifs, including a tyrosine phosphorylation site (Hatakeyama, 2004). Four distinct EPIYA sites have been described, EPIYA-A, EPIYA-B, EPIYA-C and EPIYA-D each of which is conserved in sequence. In Europe, North America, and Australia, many *H. pylori* isolates *cagA* carries EPIYA-A, EPIYA-B and EPIYA-C segments in tandem and is called Western type and

shown in figure 2.3. In East Asian countries such as Japan, Korea and China *cagA* carries EPIYA-A, EPIYA-B and EPIYA-D segments in tandem and is called East Asian type and shown in figure 2.3 (Higashi *et al.*, 2002b). The EPIYA-C segment multiplies (mostly one to three times) in tandem among different Western *cagA* species. *CagA* from East Asian *H. pylori* isolates also possesses EPIYA-A and EPIYA-B segments, but not the repeatable EPIYA-C segment. Instead, it has EPIYA-D segment, which is unique to East Asian *cagA* (Hatakeyama, 2009). The EPIYA-C segment is found in Western *cagA* and it includes hpAfrica1, hpEurope, hpNEAfrica strains and some hpAsia2 strains from Southeast Asia, while the EPIYA-D segment is unique for East-Asian *cagA* in hpEastAsia strains and in some hpAsia2 strains from Ladakh in Northern India (Olbermann *et al.*, 2010).

Majority of *H. pylori* isolates in East Asian countries possess East Asian *cagA*. In contrast, nearly all *H. pylori cagA*-positive strains isolated in Western countries carry Western *cagA* (Figure 1.3). Interestingly, in Southeast Asia countries like Malaysia, Thailand, Philippines and Vietnam (Hatakeyama, 2011). East Asian (ABD) *cagA*-carrying *H. pylori* and Western (ABC) *cagA*-carrying *H. pylori* may have been introduced into Southeast Asian people through migrations of ethnic Chinese and ethnic Indian people, respectively (Graham *et al.*, 2007; Yamaoka, 2009; Sahara *et al.*, 2012).

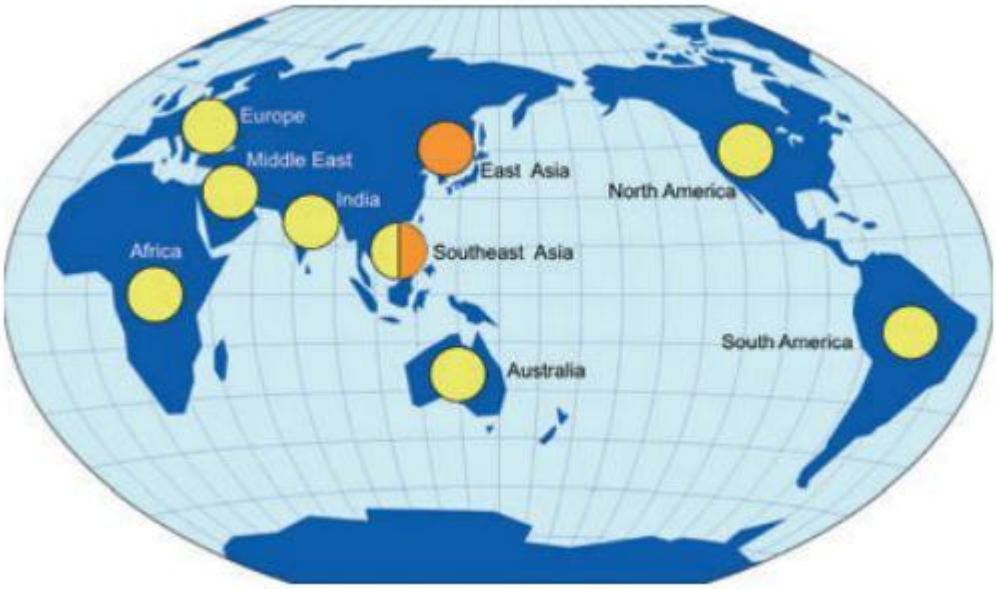

Figure 1.3 Worldwide distribution of *H. pylori* Western *cagA* and East Asian *cagA* Western *cagA* shown in yellow and East Asian *cagA* in orange colour, Adapted from (Hatakeyama, 2011).

More than 60% of Western *CagA* proteins carry the EPIYA-ABC, followed by the EPIYA-ABCC 20.3%, then the EPIYA-ABCCC 4.0% as shown in Figure 2.4 (Hatakeyama, 2011). In contrast to the EPIYA-C segment, the EPIYA-D segment seldom duplicates and thus majority of the East Asian *CagA* isolates contain a single EPIYA-D segment (98.8%), which, in many cases, is present in EPIYA-ABD (Figure 1.4) (Xia *et al.*, 2009).

EPIYA type D or multiple C repeats is associated with increased SHP-2 phosphatase activity induced by *cagA*. Strains possessing *cagA* with greater numbers of type C phosphorylation motifs predispose to precancerous lesions and gastric cancer (Azuma *et al.*, 2002; Naito *et al.*, 2006). Thus, determination of the degree of *cagA* phosphorylation or the number of phosphorylation motifs appears to be more important than detection of *cagA* alone (Argent *et al.*, 2005).

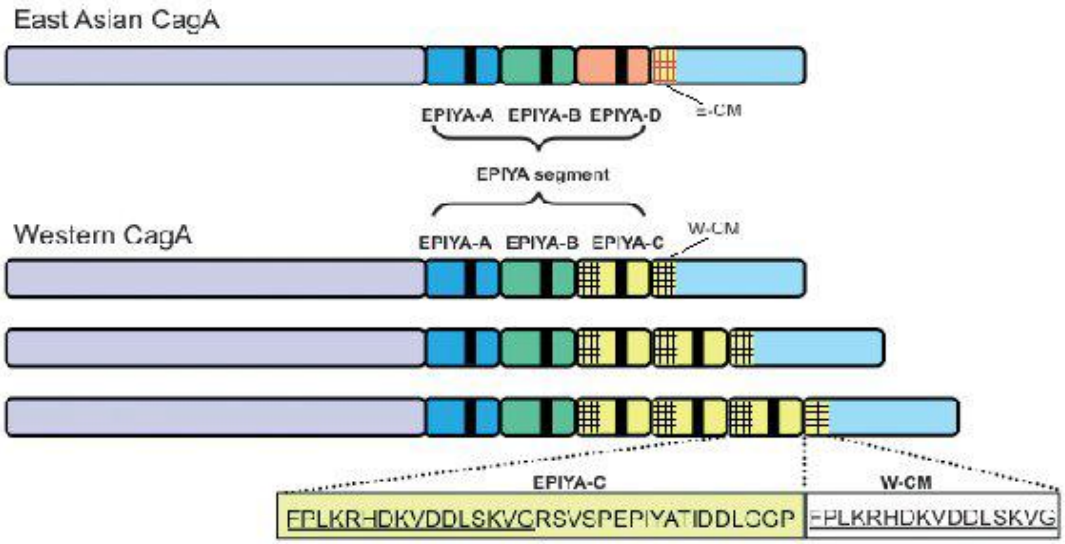

Figure 1.4 Structural polymorphisms in *cagA* and *cagA* multimerization (CM) sequence adapted from (Hatakeyama, 2011).

1.6.4 Vacuolating cytotoxin gene A (*vacA*)

The *vacA* gene is present in all *H. pylori* strains and encodes a vacuolating cytotoxin. There are at least four *vacA* signal sequence types (s1a, s1b, s1c, and s2) and two middle region types (m1, m2) (Ben Mansour *et al.*, 2010). Type s1/m1 mosaic combination strains express more cytotoxin activity than s1/m2 strains, and s2/m2 strains produce no detectable cytotoxin activity (van Doorn *et al.*, 1998; Ben Mansour *et al.*, 2010). Mature *VacA* s1-type strains has a hydrophobic N terminal region which is able to insert into the host cell membrane leading to vacuolation, whereas non-toxigenic s2- type strains have an N-terminus preceded by a hydrophilic region that blocks vacuolation (McClain *et al.*, 2001). *H. pylori vacA* type s1 strains seems to be more virulent than type s2 strains and are associated with higher risks for peptic ulcer disease, gastric atrophy, and gastric carcinoma (Figueiredo *et al.*, 2002).

There is geographical variation in the *vacA* genotypes. In Western country, including Latin America and Africa, studies have reported that individuals infected with s1 or m1 *H. pylori* strains have an increased risk of peptic ulcer or Gastric cancer compared with individuals infected with s2 or m2 strains (Sugimoto and Yamaoka, 2009). There is also a variation of m strain within East Asia; for instance, where m1 strains are common in parts of Northeast Asia, such as Japan and South Korea and m2 strains being predominant in parts of Southeast Asia, such as Taiwan and Vietnam (Yamaoka *et al.*, 2002; Uchida *et al.*, 2009).

1.6.5 Duodenal ulcer promoting gene (*dupA*)

dupA, which is located in the plasticity region of *H. pylori* genome, has been found to be a risk factor for duodenal ulcer (DU) and a protective factor against gastric cancer (GC) in Japan and Korea (Lu *et al.*, 2005). The *dupA* gene contains two continuous sequences, *jhp0917* and *jhp0918* which was initially reported to be a marker for DU development, but some studies showed that this gene can also be associated with GC (Argent *et al.*, 2007; Schmidt *et al.*, 2009a). Studies in patients from Brazil, India, China, USA, South Africa, Belgium, Iraq and Iran have confirmed that *jhp0917* and *jhp0198* are both present and form a continuous ORF, they have been unable to consistently replicate an association with gastroduodenal disease (Arachchi *et al.*, 2007; Douraghi *et al.*, 2008; Hussein *et al.*, 2008; Zhang *et al.*, 2008). A recent meta-analysis by Hussein *et al.* has concluded that the effects of *dupA* may be population specific, predisposing to DU in some populations and GC and GU in others (Hussein, 2010). Recently, *dupA* has been shown to play an important role in provoking IL-8 secretion (Queiroz *et al.*, 2011). In East Asian region this gene has been linked with the high risk of GC development. In the study that identified *cagA* gene in all samples as well suggesting that combination of these genes, may underline the high risk of GC in this area (Wang *et al.*, 2013).

1.6.6 Blood-group antigen binding adhesin (*babA*)

Attachment of *H. pylori* to the gastric epithelium is thought to be a major contributor to *H. pylori* persistence by providing access to nutrients and protection from gastric acid and mucus turnover. *H. pylori* outer membrane proteins (OMPs) have been proposed to be critical for adaptation to the host and persistent

colonisation. Indeed, the attachment is mediated by several OMPs, the best characterised of which are the Lewis(Le)b blood-group antigen binding adhesin (*babA*), the outer membrane inflammatory protein (*OipA*) and the sialic acid binding adhesin (*SabA*) (Ilver *et al.*, 1998).

BabA is a blood-group antigen-binding adhesin encoded by the *babA2* gene, which has been shown to mediate adherence of *H. pylori* to human Lewis b blood-group (Leb) antigens (Gerhard *et al.*, 1999). Although three *bab* alleles have been identified (*babA1*, *babA2* and *babB*), only the *babA2* gene product is functional for Leb binding activity (Pride *et al.*, 2001). Studies in Western countries have disclosed associations between the presence of *babA2* gene and digestive diseases such as duodenal ulcer and gastric cancer (Gerhard *et al.*, 1999). However, in Asia, most of the *H. pylori* strains are *babA2*-positive, irrespective of clinical outcome (Mizushima *et al.*, 2001; Sheu *et al.*, 2003). A study done in Iranian patients recently reported that *babA2* distribution was significantly higher in GC subjects (95%) when compared with non-ulcer dyspepsia patients (26.1%) and DU patients (Talebi Bezmin Abadi *et al.*, 2013) Thus, conclusions about the relationship between *H. pylori* genotypes and clinical outcome derived from one geographic region may not be true for other geographic regions.

1.6.7 Sialic acid-binding adhesin (*sabA*)

H. pylori OMP, the sialic acid-binding adhesin (*SabA*), binds to sialylated carbohydrate structures, which are upregulated as part of complex gangliosides in inflamed gastric tissue. The capacity to bind to the glycosylated epithelial cells is considered to be important for *H. pylori* to cause constant infection and disease

(Aspholm *et al.*, 2006; Odenbreit *et al.*, 2009). *SabA* was postulated to contribute to the chronic persistence of the infection (Mahdavi *et al.*, 2002; Aspholm *et al.*, 2006). Investigation of the role of *SabA* in disease development has gained momentum over the last few years, with evidence suggesting that *sabA* not only plays a role in disease development but that this role may be consistent across populations. For example, a study of a developing Hispanic country (Columbia) and a developed Caucasian country (USA) found a similarly increased prevalence of *sabA* in DU and GC in both populations (Yamaoka *et al.*, 2006). It has also been reported that the off status of *sabA* is associated with DU, but not GU, suggesting that *sabA* may be a reliable marker for specific disease outcome (de Jonge *et al.*, 2004). However, a more recent study in Taiwan found no association with disease (Sheu *et al.*, 2006).

1.7 *H. pylori* and clinical outcome

H. pylori infection is found to be associated with gastritis, DU, GU, non-ulcer dyspepsia, GC and gastric lymphoma of mucosa associated lymphoid tissue (MALT) (Figure 2.5). Infection usually occurs during childhood and causes symptomatic acute gastritis in most patients and persists for decades or life-long, the infection can take multiple courses. Most people infected with *H. pylori* usually show no developing symptomatic disease; however, 10-15 % will develop peptic ulcer disease, approximately 1% will develop gastric adenocarcinoma, while a small group of patients will develop gastric mucosa associated lymphoid tissue lymphoma (MALToma) (Wu *et al.*, 2008; Varbanova and Malfertheiner, 2011). A normal or high acid secretion predisposes to duodenal ulcers (DU), whereas a low acid

secretion predisposes to gastric ulcers (GU) and gastric cancer (GC) (Gerrits *et al.*, 2006) as indicated in Figure 1.5.

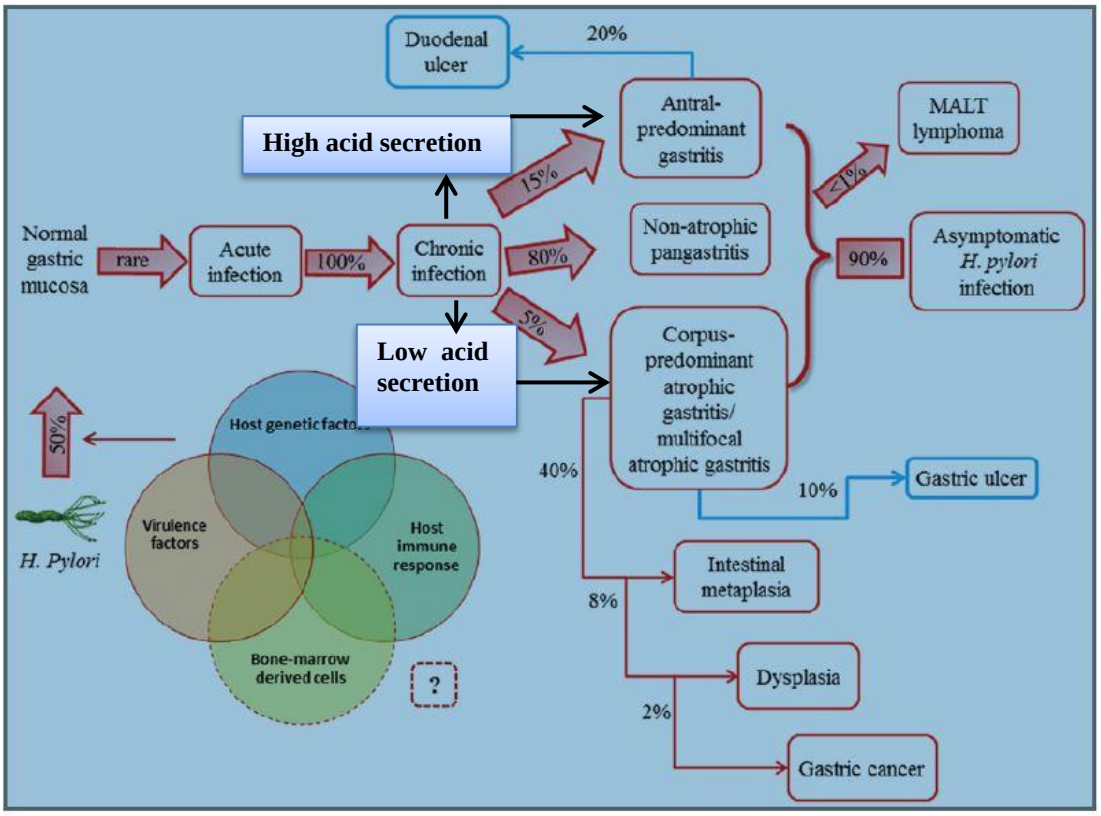

Figure 1.5 Modified natural history of *H. pylori* infection adapted from (Conteduca *et al.*, 2013).

Patients with increased acid secretion are likely to have antral-predominant gastritis, which predisposes to duodenal ulcers. Patients with low acid secretion will more likely develop gastritis in the body of the stomach and are thus more likely to develop gastric ulcer, leading to gastric atrophy, intestinal metaplasia, dysplasia and in rare cases, gastric carcinoma. This sequence of events is more frequent in people of advanced age. *H. pylori* infection induces the formation of mucosa-associated lymphoid tissue (MALT) in the gastric mucosa and MALT lymphoma is another rare complication of *H. pylori* infection (Figure 1.5).

The gastric mucosa is well protected against bacterial infections. *H. pylori* is adapted to this ecologic niche, with a array of features that permit entry into the mucus, swimming spatial orientation in the mucus, attachment to epithelial cells, evasion of the immune response and as a result persistent colonization and transmission (Suerbaum and Michetti, 2002). After being ingested, the bacteria have to evade the bactericidal activity of the gastric luminal contents and enter the mucous layer. Urease production and motility are essential for this first step of infection. Urease hydrolyzes urea into carbon dioxide and ammonia, thereby permitting *H. pylori* to survive in an acid milieu (Weeks *et al.*, 2000; Dhar *et al.*, 2003).

1.7.1 Gastritis

Gastritis refers to inflammation of the gastric mucosa. When a person is infected with *H. pylori* the initial response to infection is the development of an acute gastritis. This acute phase is characterized by the presence of fever, vomiting, nausea

and mucosal inflammation, followed by cramping, gastric pain, and the development of hypochlorhydria (Kusters *et al.*, 2006). The majority of *H. pylori* infected individuals are asymptomatic despite the ongoing gastritis and if *H. pylori* is not eliminated, the acute gastritis progresses to chronic gastritis (Kusters *et al.*, 2006).

In the infected host with intact acid secretion, *H. pylori* inhabits the antral region of the stomach where few acid secretory parietal cells are localized. Antral-predominant gastritis develops to match this colonization phenotype. In the case of chronic infection, there is a loss of parietal and chief cells in the oxyntic glands, resulting in a reduction in acid secretion known as hypochlorrhydria. In cases where acid secretion is impaired due to parietal cell loss, or inhibition of parietal cell function, bacteria are distributed between both the antrum and the corpus (Kusters *et al.*, 2006). Of those infected, a small proportion of patients infected with *H. pylori* will develop more serious clinical outcomes such as peptic ulcer disease (PUD), gastric cancer and MALT lymphoma (Kusters *et al.*, 2006).

1.7.2 Peptic ulcer disease (PUD)

Peptic ulcer disease (PUD) grows when the protective mechanisms of the gastrointestinal mucosa, such as mucus and bicarbonate secretion, are overwhelmed by the damaging effects of gastric acid. Peptic ulcers occur mainly in the stomach and it is refered to as GU or proximal duodenum and refered to as DU. *H. pylori* infection was originally identified as the main cause of PUD; however, as the prevalence of *H. pylori* infection has declined, GU has been more commonly associated with the use of nonsteroidal anti-inflammatory drugs (NSAIDs) (Yuan *et al.*, 2006; Ramakrishnan and Salinas, 2007).

DU only develops when *H. pylori*-associated inflammation arises mostly in the antrum. *H. pylori* is present in the gastroduodenal mucosa in most patients with especially duodenal ulcers, however, only a minority (10 to 15 percent) of patients with *H. pylori* infection develop peptic ulcer disease (Ramakrishnan and Salinas, 2007; Wu *et al.*, 2008). DU is usually associated with an antral predominant gastritis and a high acid output. In high acid secretors there is an increased output of acid to the duodenum, which initiates the replacement of intestinal cells with gastric epithelial cells, known as gastric metaplasia. In turn, this metaplasia provides an appropriate environment for *H. pylori* to colonise the duodenum and cause an active chronic duodenitis, that may predispose the individual to DU (Blaser and Atherton, 2004; Egan *et al.*, 2007).

Gastric ulcer is also associated with diffuse gastritis. *H. pylori*-related gastric ulcer is found most frequently at the transitional zone between antrum and corpus on the lesser curve. The dense colonization of *H. pylori*, intense inflammation, and intestinal metaplasia, and the subsequent epithelial damage leads directly to ulceration in this region. GU is associated with corpus predominant gastritis with low acid output (Shanks and El-Omar, 2009).

PUD leads to the most common complication like bleeding. This occur in about 10–20% of patients with *H. pylori* associated PUD and is the most common cause of nonvariceal upper gastrointestinal bleeding. Bleeding of peptic ulcer is a complication with major morbidity and mortality (de Vries and Kuipers, 2010).

The second most common cause of peptic ulcer is non-steroidal anti-inflammatory drugs (NSAID) and aspirin use. Aspirin appears to be the most

ulcerogenic of NSAIDs (Furuta and Delchier, 2009). NSAIDs or *H. pylori* alone increase the risk of peptic ulcer about 20-fold, but those who were *H. pylori* infected and NSAID users are 60 times more likely to develop a peptic ulcer than uninfected non-NSAID users (Lai and Sung, 2007). NSAIDs and *H. pylori* infection independently increase also the risk of peptic ulcer bleeding. In patients with chronic NSAID use, PPI maintenance treatment is better than *H. pylori* eradication therapy in preventing upper gastrointestinal bleeding. However, *H. pylori* eradication therapy is of value in chronic NSAID and low dose aspirin users although it is insufficient to prevent ulcer disease and bleeding completely (Lanas, 2010). *H. pylori* infection, PUD and NSAID use have led to upper gastrointestinal bleeding particularly the elderly population. As elderly patients often have more comorbidities and more complicated PUD, they may also experience worse outcomes than young PUD patients (Musa *et al.*, 2012).

1.7.2.1 Complications of peptic ulcer

The complications of peptic ulcers include haemorrhage, obstruction, and perforation. Haemorrhage is caused by bleeding from granulation tissue or from erosion of an ulcer into an artery or vein. Bleeding may be manifested by hematemesis or melena. Bleeding may be sudden, severe, and without warning. Bleeding from peptic ulcers is strongly associated with NSAID and aspirin use (Quan *et al.*, 2014).

1.7.3 MALT(mucosa- associated lymphoid tissues) Lymphoma

H. pylori is also associated with the development of MALT lymphoma, there is convincing evidence that gastric MALT lymphoma is caused by *H. pylori* infection (Du, 2007). Studies have shown that the growth of the lymphoma B-cells can be stimulated by intratumoral *H. pylori* specific T- cells, involving direct B and T cell interaction through surface costimulatory molecules. In the majority of patients receiving *H. pylori* eradication therapy, complete disappearance of gastric MALT lymphoma has been found (Nakamura *et al.*, 2001; Du, 2007).

1.7.4 Gastric cancer

Despite the latest decline in incidence and mortality over the past few decades, GC is the fourth most common cancer and second leading cause of cancer related death worldwide (Fox and Wang, 2007). Two-thirds of stomach cancers occur in developing countries, with the highest incidence reported in Japan and Korea (Yamamoto, 2001; Crew and Neugut, 2006). Gastric cancer can be classified into cardia gastric cancer (CGC) or proximal GC and non-cardia gastric cancer (NGC) or distal GC. Gastric cancer develops in 2.9% of *H. pylori* infected Patients. *H. pylori* infection is liable for about 75% of all non-cardia gastric cancers and 63.4% of all stomach cancers worldwide (Uemura *et al.*, 2001; Wen and Moss, 2009). Several studies have shown that an association exist between *H. pylori* and NGC but not CGC (Fox and Wang, 2007).

In 1994, on the basis of various epidemiological studies, *H. pylori* was classified as a class I carcinogen in humans by a working group of the World Health Organization International Agency for Research on Cancer (IARC, 1994). Although *H. pylori* infection is considered the single most important risk factor, it is believed that other factors like environment, diet, genetics, host, bacterial or viral infections contribute to the malignant progression of the gastric cancer (Figure 1.6).

The carcinogenic effect of *H. pylori* can be improved by dietary and environmental factors. *H. pylori* infection is more common in less developed Asian countries (*e.g.*, India, Bangladesh, Pakistan, and Thailand) in comparison with the more developed Asian countries (*e.g.*, Japan and China). However, the frequency of gastric cancer is paradoxically very low in these less developed regions than in Japan and China (the so-called "Asian enigma) (Singh and Ghoshal, 2006).

A case control study in Japan showed that young adults between the age range of 20 to 39 infected with *H. pylori* had an odds ratio of 13.3 for the development of GC as compared with those not infected (Kikuchi *et al.*, 1995). In another large scale prospective study reported by Uemura et al, 36 (2.9%) of 1246 of *H. pylori* infected subjects developed GC over a 7.6 years follow-up period as compared with 0% of the 280 non-infected subjects (Uemura *et al.*, 2001).

Many studies have also shown that *H. pylori* eradication therapy can result in the regression of gastric atrophy and intestinal metaplasia hence showing that there is an association between *H. pylori* and GC (Ohkusa *et al.*, 2001; Ito *et al.*, 2002; Leung *et al.*, 2004). A recent metaanalysis showed that there was 35% reduced risk of gastric cancer in those who were treated for *H. pylori (*Fuccio *et al.*, 2009*)* In

addition, a randomized study with early gastric cancer showed a 65% reduction in the incidence of metachronous gastric cancers in those who were treated for *H. pylori (*Fukase *et al.,* 2008*).*

Thirty years ago, Correa *et al,* (1976) proposed that gastritis could progress to gastric cancer, which made the identification of *H. pylori* as a major cause of gastritis even more relevant. Chronic *H. pylori*-induced inflammation can eventually lead to loss of the normal gastric mucosal architecture, with destruction of gastric glands and replacement by intestinal metaplasia. This process of atrophic gastritis and intestinal metaplasia occurs in approximately half of the *H. pylori*-colonized population, first in those subjects and at those sites where inflammation is most severe (Kuipers *et al.*, 1995). The presence of *cagA* has been associated with peptic ulcer disease and gastric cancer (Miehlke *et al.*, 2001).

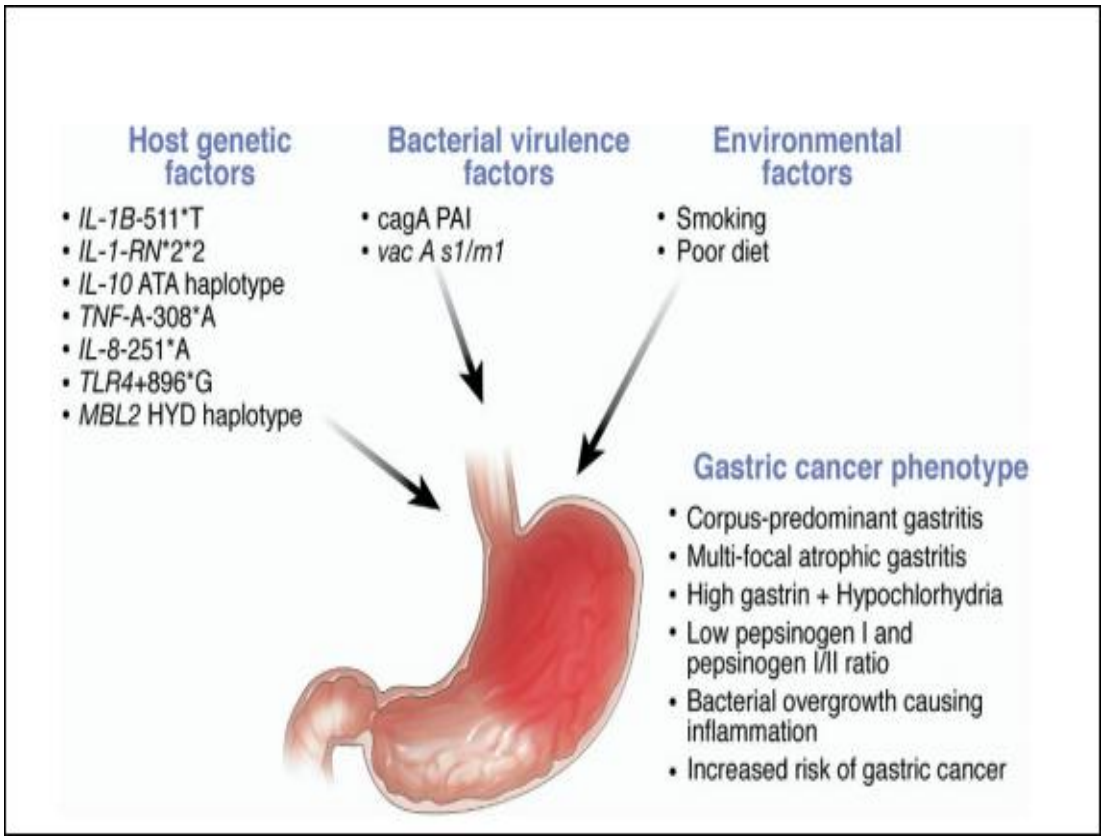

Figure 1.6 Contribution of host genetic, bacterial, and environmental factors to pathogenesis of *H. pylori*-induced gastric cancer. These factors combine to create an intragastric milieu characterized by chronic inflammation, hypochlorhydria, bacterial overgrowth, and sustained genotoxic stress. This could ultimately lead to gastric adenocarcinoma adapted from (Amieva and El-Omar, 2008).

1.7.5 The enigmas

Studies have shown geographical- or population-associated enigmas, such as African, Asian, and Indian enigmas; in such enigmas, the observations of a particular population or geographic region do not correlate with the expected outcomes (Miwa *et al.*, 2002; Goh *et al.*, 2007).

1.7.5.1 Asian and East Asian enigmas

Asian enigma refers to the concept stating that the prevalence of *H. pylori* infection in countries, such as Bangladesh and Pakistan, is high and the rate of GC is low; *H. pylori* infection is also prevalent in other countries, such as Korea, Japan, and China, but the rate of GC is high (Miwa *et al.*, 2002). Indeed, the prevalence of *H. pylori* or virulent strains and the incidence of GC are inconsistent. In Linqu County, China, the incidence of GC is extremely high; in its neighbouring county Cangshan, the incidence of this condition is very low (Dong *et al.*, 2012). However, the rate of *H. pylori* infection and the proportion of virulent strains in these two counties are not significantly different (Groves *et al.*, 2002). The ancestral origin of the bacterial species can be a significant factor that contributes to gastric carcinogenesis. This conclusion is supported by a previous study conducted in Malaysia (Tay *et al.*, 2009), where three ethnic groups have been identified: Malay, Indian, and Chinese. The infection rate of *H. pylori* differs between ethnic groups; for example, Malays exhibit lower infection rate than Indians and Chinese subjects (Goh and Parasakthi, 2001). However, the incidence of GC is similar to Malays and Indians, but this incidence is much lower than that in Chinese subjects (Goh *et al.*, 2007).

1.7.5.2 Indian enigma

The Indian enigma, part of the Asian enigma, was first described in ethnic Indians residing in Malaysia; this enigma is related to the low prevalence of GC despite the high prevalence of *H. pylori* infection (Goh *et al.*, 2007). DU is common in numerous regions in India and considered as common among migrants from an Indian subcontinent (Graham *et al.*, 2009). GU and GC are comparatively rare in India, although these outcomes are mutually exclusive. These enigmas are possibly attributed to the interaction of host genetic factors and environmental factors, such as diet, rather than *H. pylori* strain (Goh *et al.*, 2007; Graham *et al.*, 2009). The varied clinical outcomes of *H. pylori* infection may be influenced by dietary habits of study populations. Diets rich in antioxidants, such as fresh fruits and vegetables, decrease the risk of gastric cancer; this result indicates that oxidative stress and chronic inflammation may be among the mechanisms by which *H. pylori* induces gastric cancer. Nevertheless, curcumin, an active anti-inflammatory compound in Indian spices, can inhibit NF-κB complex activation and IL-8 induction in gastric cells infected with *H. pylori* (Foryst-Ludwig *et al.*, 2004; Bengmark, 2006). People living in the Indian subcontinent consume these ingredients daily from a very young age.

1.7.5.3 Africa enigma

The African enigma is explained by several reports from different areas; this concept confirms high infection prevalence and low gastric cancer frequency (Holcombe, 1992). However, the concept of an African enigma was challenged almost as soon as it was proposed. Studies have opposed this claim because data were obtained from populations with extremely limited access to health care and relatively short life

expectancies. Furthermore, the prevalence rate of peptic ulcer disease among those with *H. pylori* infection is similar to that recorded in many developed countries (Henriksen, 2001; Agha and Graham, 2005). Therefore, the African enigma is considered as a medical myth.

1.8 Diagnosis of *H. pylori* and treatment

1.8.1 Diagnosis of *H. pylori* infection

Accurate diagnosis of *H. pylori* is important both in routine clinical practice and in clinical research (Wong *et al.*, 2001). *H. pylori* can be diagnosed by variety of invasive and non-invasive tests. Invasive tests are based on gastric samples, usually mucosal biopsies, which can be screened by rapid urease test, histology, culture and polymerase chain reaction. Non-invasive tests required alternative clinical specimens, for example, blood, faeces, urine, or saliva. These samples can be screened by serology, urea breath test and stool antigen test (Moss and Sood, 2003; Versalovic, 2003). The choice of an appropriate test is based on its performance and the clinical situation, as well as economic considerations (Moss and Sood, 2003).

1.8.1.1 Invasive tests

1.8.1.1.1 Culture

Culture is the gold standard for diagnosis of *H. pylori* infection, it is 100% specific but its sensitivity is low (Brown, 2000). Isolation of *H. pylori* from gastric

biopsy specimen is a difficult procedure that is affected by many factors, such as, the duration of time between tissue collection and culture, type of transport media, transport temperature and duration of exposure to air (Ndip *et al.*, 2003; Yuen *et al.*, 2005). *H. pylori* isolation and sample processing requires an experienced laboratory hence a better result. Culture is necessary for study of bacterial virulence factors and antibiotic susceptibility testing (Rautelin *et al.*, 2003; Zullo *et al.*, 2003).

1.8.1.1.2 Histology

Histology is considered to be important in the diagnosis of *H. pylori* infection (Malfertheiner *et al.*, 2012). *H. pylori* has spiral appearance that can be identified during histological examination. A wide range of staining like Hematoxylin-Eosin and modified Giemsa can be used. Histology is important as it gives information about the grade of inflammation in the gastric mucosa by evaluating the infiltration of granulocytes and lymphocytes as well as presence of gastric atrophy and intestinal metaplasia.

The sensitivity of *H. pylori* diagnostic tests has been known to reduce in patients with peptic ulcer bleeding, but Choi et al, reported histology is not affected by bleeding patients and provides reliable result regardless of the presence of bleeding (Choi *et al.*, 2012). In addition, a meta-analysis study reported that histology has a higher sensitivity and specificity when compared with the UBT and the RUT for the diagnosis of *H. pylori* infection after a partial gastrectomy.

1.8.1.1.3 Rapid urease test (RUT)

The rapid urease test is a reliable, simple and cost-effective method for the detection of *H. pylori* (Granstrom *et al.*, 2008). Rapid urease testing takes advantage of the fact that *H. pylori* is a urease-producing organism. The principal of the test is based on the activity of the *H. pylori* urease enzyme that splits urea into carbon dioxide and ammonia resulting in an increase of pH which is detected by the color change of a pH indicator to red. Some commercially available tests include the CLO test, hpfast and Pylori Tek (Vaira *et al.*, 2002). RUTs are considered highly accurate with a sensitivity and specificity over 90% (Kuo *et al.*, 2002; van Keeken *et al.*, 2006), although in an active ulcer bleeding, the sensitivity of RUT may be reduced (Choi *et al.*, 2012).

1.8.1.2 Non-invasive tests

1.8.1.2.1 Urea Breath test (UBT)

The urea breath test (UBT) is a reliable, simple, non-invasive technique that not only allows for *H. pylori* diagnosis but is also useful in confirmation of eradication after treatment (Gisbert and Pajares, 2004a). The sensitivity and specificity of the 13C-Urea Breath Test (UBT) are in the range of 97% and 95%, respectively in both untreated and treated patients, However, it is not constantly available in normal clinical settings, moreover it requires expensive equipment (Vaira *et al.*, 2002; Falsafi *et al.*, 2009). The 13C UBT identifies gastric *H. pylori* urease activity by measuring 13C enrichment in expired breath samples after

ingestion of 13C-labelled urea. Two breath samples are collected, one before and another 30 min after urea ingestion, regardless of the dosage of the isotope administered (Mauro *et al.*, 2006). The principle of the UBT depends on the capability of *H. pylori*, when present in the stomach, to hydrolyse orally administered labelled urea to produce isotopically labelled CO_2 which diffuses into the blood, is excreted by the lungs and can be identified in breath samples by means of measuring equipment (Gisbert and Pajares, 2004a).

Urea can be labelled with two different isotopes, 14C (the radioactive isotope) or 13C (the non-radioactive stable isotope). Labelling urea with 13C has become increasingly popular because the non-radioactive isotope is safe and can also be safely performed in children, pregnant women, and women of child-bearing age (Vaira *et al.*, 2002).

1.8.1.2.2 Serology

Serological tests were based on the detection of a specific anti-*H. pylori* IgG antibody in patient's serum. Serology is a suitable method to diagnose *H. pylori* infection, especially in large scale epidemiological studies and in screening protocols because it is easy to perform and non-invasive (Hoang *et al.*, 2004). However, they have limitations. The most important one is the inability to differentiate between active infection and previous contact. Antibody levels remain in the blood for long period of time and as more patients with *H. pylori* infection are treated, persistent antibody will lead to false positive results (Vaira *et al.*, 2002). The ELISA method and the Western blot method are used to detect the antibodies against *H. pylori* in the patient's serum.

1.8.1.2.3 Stool antigen test

The stool antigen test is considered as a valuable noninvasive alternative to diagnose *H. pylori* when UBT is not available. These tests are based either on monoclonal or polyclonal antibodies. They include HePy-stool, Hp Ag, Amplified IDEIA HpStAR and Premier Platinum HpSA) and commercial near patient tests like ImmunoCard STAT HpSA have also been developed. The most studied tests are the polyclonal antibody-based HpSA, the monoclonal antibody-based HpStAR, and the immunochromatographic ImmunoCard based on monoclonal antibodies (Gisbert *et al.*, 2004; Veijola *et al.*, 2005). It is suitable both for primary diagnosis and monitoring treatment outcome. A sensitivity of 91% and specificity of 94% were reported in previously untreated individuals (Gisbert and Pajares, 2004b).

1.8.1.3 Molecular Methods

1.8.1.3.1 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) allows detection of the organism in small samples with few bacteria present and without special requirements in processing and transport. Furthermore, PCR can be performed rapidly and cost-effectively, and it can be used to identify different strains of bacteria for pathogenic and epidemiologic studies. The major constraint of PCR is that relatively few laboratories currently have the capability to run the assay (Hardin and Wright, 2002). PCR has been used to amplify and detect *H. pylori* specific genes responsible for its

pathogenicity. The test's sensitivity is over 90% and its specificity is almost 100% (Ciacci *et al.*, 2004).

PCR has been used extensively for the diagnosis of *H. pylori* from gastric biopsy specimens, gastric juice, saliva, faeces, dental plaque and archival specimens, as well as for detecting clarithromycin resistance. PCR yields information on the presence of potential virulence markers in the strain which might have implications for the development of severe disease (Ricci *et al.*, 2007; Rimbara *et al.*, 2013). PCR-based methods also enable identification of antimicrobial resistance to clarithromycin (Chisholm *et al.*, 2001; Oleastro *et al.*, 2003). The basic technique of PCR includes repeated cycles of amplifying selected nucleic acid sequences. Each cycle consists of the following steps:

DNA denaturation step: This stage involves heating the reaction to 94-98 ^{o}C for 20-30 seconds. The heating leads separation of double strands DNA by disrupting hydrogen bonds between complementary bases.

Primer annealing step: This is performed at a lower temperature of 50-65 ^{o}C for 20-30 seconds. This process will anneal the primers to a single stranded DNA template.

An extension reaction step: The temperature in this step depends on DNA polymerase used. *Taq* polymerase enzyme uses a temperature of 72 ^{o}C. In this step the DNA polymerase synthesizes a new DNA strand complementary to the DNA template stand by adding DNTPs that are complementary to the template in 5′ to 3′ direction leading to extension of DNA strand.

Final elongation: This single step is occasionally performed at a temperature of 70 to 74 ^{o}C for 5-15 min after the last PCR to ensure that any remaining single stranded DNA is fully extended.

At the end of each cycle (each consisting of the above three steps), the quantities of PCR products are theoretically doubled. The whole procedure is carried out in a programable thermal cycler. Generally, performance of between 30 to 50 thermal cycles results in an exponential increase in the total number of DNA copies synthesized adequate for most studies (Tang *et al.*, 1997).

1.8.1.3.2 Whole genome microarrays

DNA microarrays have been broadly used to determine genome-wide variations in terms of presence and absence of genes. Microarray technology has an advantage of being able to analyse the presence and absence of thousands of genes simultaneously (Lockhart and Winzeler, 2000).

Genome Wide Human SNP Array 6.0 platform offers the genotype calling algorithm "Birdseed" to determine the genotypes of 906,600 SNPs and contains 945,826 copy number probes designed to interrogate (copy number variation) CNVs in the genome. The Genome-Wide Human SNP Array 6.0 thus provides a flexible, cost-effective method for scoring SNP genotypes in large numbers of samples (http://www.affymetrix.com/index.affx).

DNA microarray “chips” although small-sized, they contain a dense, regular array of DNA oligonucleotides designed to probe the presence of over a million

alleles distributed throughout the genome (Distefano and Taverna, 2011). The advent of DNA microarrays allows the analysis of gene alterations across the entire human genome. DNA samples were genotyped using the Genome Wide Human SNP 6.0 array. Genomic DNA samples were at the beginning digested with *Nsp* I and *Sty* I restriction enzymes and then adaptor-ligated and PCR amplified using a primer that recognizes the adaptor sequence. PCR products were then purified, labeled, fragmented and hybridized to oligonucleotide probes fixed to the array. This was followed by washing and staining process and finally the sample scanned (Figure 1.7) (Mijatovic *et al.*, 2012).

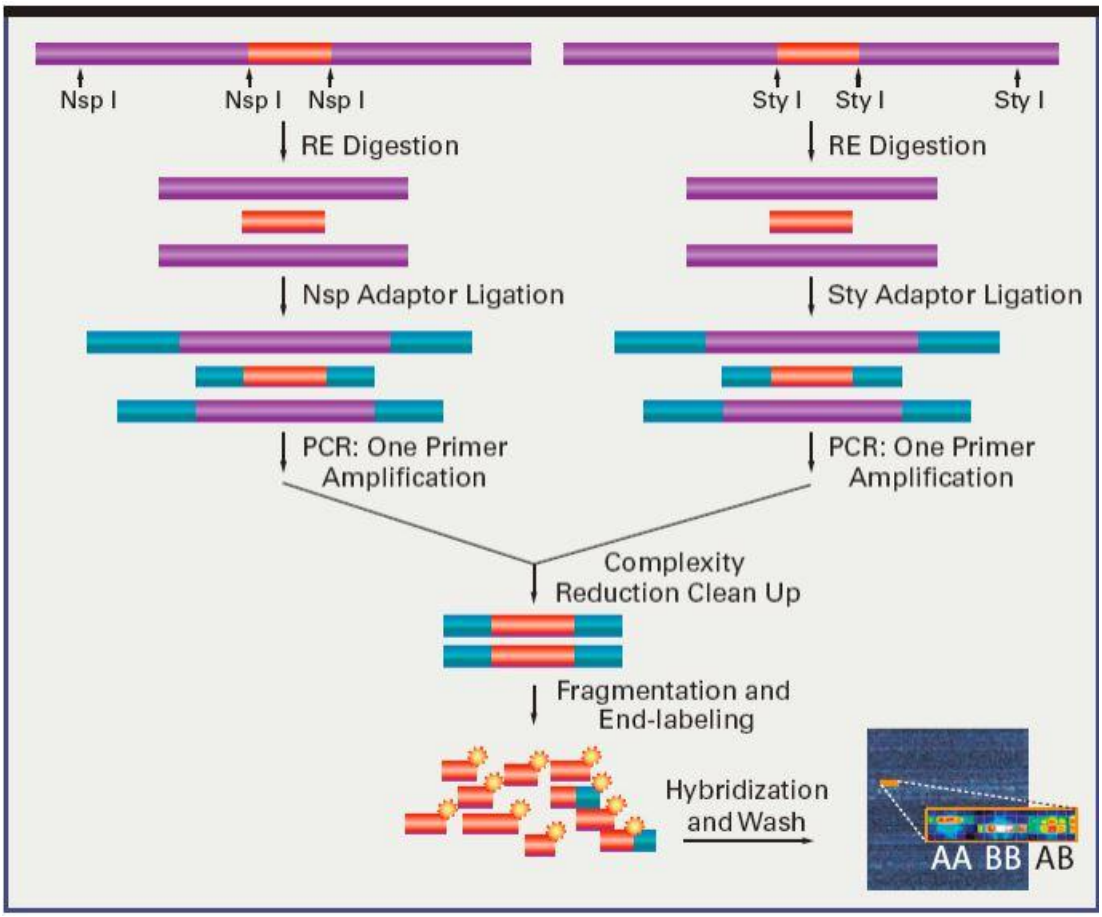

Figure 1.7 Genome wide SNP 6.0 assay overview (Genome-Wide SNP 6.0 Manual)

1.8.1.4 *H. pylori* treatment

Antibiotic resistance has become a world-wide concern (Westh *et al.*, 2004). In recent years, there has been high cases of multiple resistance in many microorganisms that are human pathogens, mainly as a result of the indiscriminate use of the antimicrobial drugs. The prevalence of antimicrobial resistance varies with geographical region due to strict rules in some areas (Ndip *et al.*, 2008). Data on antibiotic susceptibility and resistance in *H. pylori* highlighted regional differences in resistance patterns for clarithromycin and metronidazole (Megraud, 2004). These differences hinder the success of eradication therapy. Treating *H. pylori* infection is therefore a concern.

The infection of *H. pylori* has a high morbidity rate, but a low mortality rate, and is cured with antibiotic therapy. First-line treatment, which is a one-week triple combination therapy comprising twice daily use of a proton pump inhibitor (PPI) and two antibiotics (clarithromycin and amoxicillin or metronidazole) has been used for long (Georgopoulos *et al.*, 2013). Due to failure treatment and emergence of resistance to both clarithromycin and metranidazole, an alternative first-line treatment regimes which includes sequential therapy regimes where a PPI and amoxicillin are used for the first 5 days, metronidazole and clarithromycin replacing amoxicillin for a further 5 days have emerged (Agudo *et al.*, 2010; Georgopoulos *et al.*, 2013). Eradication of the organism has been shown to result in ulcer healing, prevention of peptic ulcer recurrence and may also reduce the prevalence of gastric cancer in high risk populations (Sepulveda and Coelho, 2002; Tanih *et al.*, 2009).

1.9 Immune responses to *H. pylori*

H. pylori infection triggers an immune response characterised by inflammation of the gastric mucosa. *H. pylori* induces a Th1-polarized response that does not result in clearance of the infection. *H. pylori* is thought to manipulate the host immune response and inflammation. The key activator of the innate immune response is probably intracellular peptidoglycan (Kusters *et al.*, 2006). *H. pylori* is capable of inhibiting phagocytosis by macrophages by unknown mechanism. IL-10-producing T cells are crucial in the control of inflammation and they enable the bacteria to persist in the gastric mucosa (Kusters *et al.*, 2006). Numerous cytokine genes have stable polymorphisms which are known to affect the level of cytokine production in response to *H. pylori* infection (El-Omar, 2006; Kusters *et al.*, 2006). The most known geses is IL-1β, a potent pro-inflammatory cytokine and the most potent known inhibitor of acid secretion (Kusters *et al.*, 2006). These cytokine polymorphisms may contribute to the risk of gastric adenocarcinoma, but their contribution to the risk of peptic ulceration is conflicting (Chakravorty *et al.*, 2006; Robinson *et al.*, 2007). Levels of cytokines interferon-γ (IFN-γ), tumour necrosis factor-α (TNF-α), IL-1, IL-6, IL-7, IL-8, IL-10, and IL-18 are reported to increase in the stomach of *H. pylori*-infected humans compared to uninfected humans (Moyat and Velin, 2014).

1.10 Host genetic factors

It is well recognized that the development of gastric disease is strongly influenced by host genetic factors. Single nucleotide polymorphisms (SNPs) play an important role in *H. pylori* induced disease. An association of increased risk of

gastric cancer and IL-IB and IL-IRN genes has been established (Ryberg *et al.*, 2008).

Interleukin-10 (IL-10) is a multifunctional cytokine with both immunosuppressive and antiangiogenic functions. IL-10 is an important immunoregulatory cytokine mainly produced by activated T cells, monocytes, B cells and thymocytes. As an immune response modulator, IL-10 can both stimulate and suppress the immune response (Mocellin *et al.*, 2005). Numerous studies have shown the controversial role of IL-1, TNF-A, 1L-1RN, IL-10 and IL-2 gene polymorphisms in the development of gastric cancer (El-Omar *et al.*, 2003; Kim, 2007).

The level of gastric acid secretion and the presence of a pro-inflammatory response contribute drastically to the development of either duodenal ulcer disease or atrophic gastritis. The IL-1 cytokine is encoded by a gene cluster that contains the polymorphic IL-1B (encoding the IL-1-β cytokine) and IL-RN (encoding the IL-1 receptor antagonist) genes. IL-1 is a potent pro-inflammatory cytokine and the most potent known inhibitor of acid secretion (El-Omar *et al.*, 2000). The IL-1 gene cluster contains several polymorphisms, such as IL-1B-31C, IL-1B-511T, and IL-1RN 2/2, which lead to high-level expression of IL-1-β. IL-1-β.is also a potent acid suppressor and acts on parietal cells directly and indirectly to inhibit gastric acid secretion facilitating the spread of *H. pylori* from the antrum to the corpus. This consequently leads to reduced acid output, which is associated with corpus-predominant colonization by *H. pylori*, resulting in pangastritis, formation of atrophic gastritis, and increased risk of gastric cancer (El-Omar *et al.*, 2000; Hwang *et al.*, 2002; Zambon *et al.*, 2002).

1.11 Human genetic variation

Human genetic variation can be defined as differences between individuals and populations at a genetic or DNA level. Studies on human genetic variation have provided insights into the genetic contributions to human disease. For instance, researchers can identify and describe the causes of several diseases based on genetics. The human genome consists of 23 pairs of chromosomes, in which one pair is inherited from each parent; these chromosomes contain approximately six billion nucleotides. Human genome sequencing revealed that any two unrelated individuals are 99.9% identical at a genetic level (Frazer *et al.*, 2009). The 0.1% difference is referred to as genome variation. This variation in the human genome consists of single nucleotide polymorphisms (SNPs), variable number of tandem repeats (VNTRs), small insertions and deletions (INDELs), and copy number variations (CNVs) (Frazer *et al.*, 2009) as indicated in the Figure 1.8. These differences are accounted for individuality. These variations also determine eye color, height, intelligence quotient (IQ), and several other traits. Unfortunately, these differences in the human DNA may lead to disease predisposition.

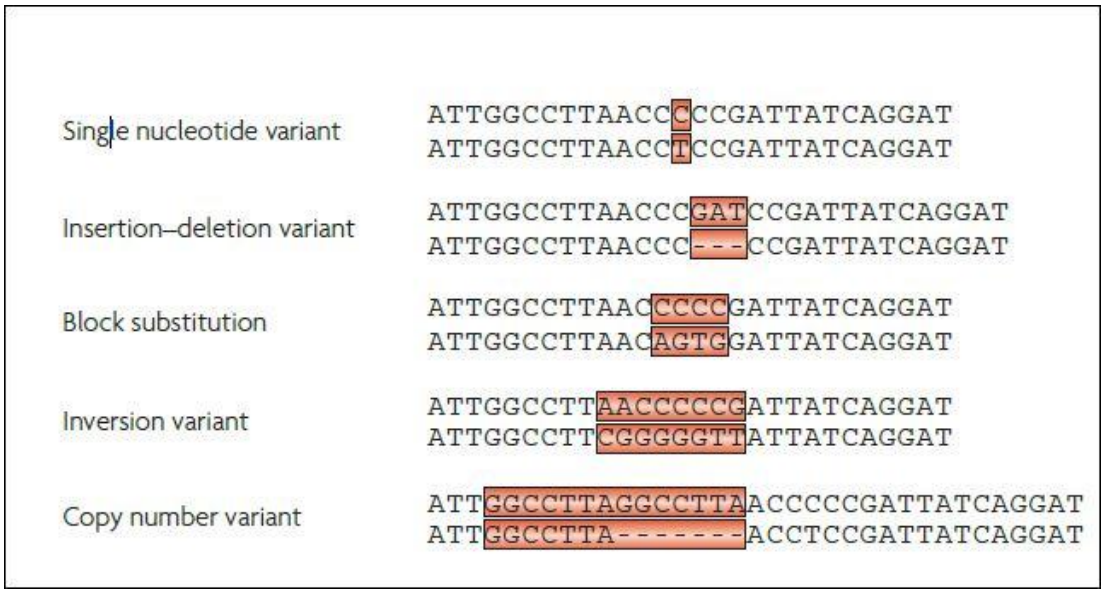

Figure 1.8 Classes of human genetic variants adapted from (Frazer *et al.*, 2009)

Single nucleotide variants are DNA sequence variations in which a single nucleotide (A, T, G or C) is altered. Insertion–deletion variants (indels) occur when one or more base pairs are present in some genomes but absent in others. Block substitutions describe cases in which a string of adjacent nucleotides varies between two genomes. An inversion variant is one in which the order of the base pairs is reversed in a defined section of a chromosome. Copy number variants occur when identical or nearly identical sequences are repeated in some chromosomes but not others.

1.11.1 Single Nucleotide Polymorphism (SNPs)

Millions of SNPs have been identified after the human genome project was completed. SNPs are the most common form of DNA variation present in the human genome; these variations occur at a frequency of >1% in human population. SNPs can occur at any locus on the genome, particularly in non-coding regions (Brookes, 1999; Zhou and Wong, 2007).

SNPs are nucleotide variants in the genome in which individual DNA sequences differ by a single base pair. For example, some DNA molecules may contain a thymine-to-adenosine (T/A) base pair change at a particular site, whereas other DNA molecules in the same population may show a guanine-to-adenosine (G/A) base pair change at the same site. These variants might lead to disease predisposition by modifying a gene's function; these variants can also be used as genetic markers to detect nearby disease-causing mutations (Pearson *et al.*, 2007).

SNPs identified in genes are important to researchers because these polymorphisms may elicit a functional effect on gene products. On average, human DNA contains one SNP in every 300 nucleotides; approximately ten million of these polymorphisms are present among three billion nucleotides in a haploid genome. SNPs can also be used as biological markers to help locate disease-causing regions in the genome. Approximately ten million SNPs have been found in the human genome (LaFramboise, 2009). Thus far, more than three million SNPs that are commonly present in humans have been successfully genotyped in 270 population samples (Johnson *et al.*, 2008).

1.11.2 Variable number of tandem repeats (VNTRs)

Variable number of tandem repeats are defined as a short sequence of nucleotides that are repeated in tandem at a specific location in the genome. An example of this is when a tri-nucleotide sequence of 'GCT' may be repeated in tandem 25 times in one individual's DNA and 28 times at the same locus in another individual. Due to their high polymorphic content, VNTR is a useful tool in linkage analysis, forensic identification, paternity testing and population genetic studies (Pakzad *et al.*, 2014).

1.11.3 Insertions and deletions (INDELs)

Insertions and deletions (INDELs) are another type of genetic variation. INDELs are the addition or removal of a small number of nucleotides from a DNA sequence. These polymorphisms are not as frequent as SNPs in the human genome (Mills *et al.*, 2006).

1.11.4 Copy number variations (CNVs)

CNVs, is as one type of genetic variation in which a large sequence of nucleotides is repeated in tandem multiple times to a variable extent among different individuals of one population (Levy *et al.*, 2007). CNVs can be caused by genomic rearrangements such as deletions, duplications, inversions and translocations.. Although CNVs is a widespread and common phenomenon in humans, some of them have been associated with susceptibility or resistance to disease (Stankiewicz and Lupski, 2010).

1.12 Genome-Wide Association Studies (GWAS)

GWAS is conducted to identify the relationship between a genetic variant and a phenotype. A GWAS is also defined as a genetic association study in which a high density of markers are genotyped; these markers should be spaced equally across all 23 chromosomes. These markers should also be in sufficient linkage disequilibrium to represent common genetic variations among individuals in study population. A sufficient number of individuals should also be genotyped (cases and controls) to obtain enough power to detect variations with slight effects that are common in complex diseases.

A GWAS hypothesis states that common variations in the human genome are responsible for common polygenic diseases (common disease-common variant model). With the HapMap project (Thorisson *et al.*, 2005), these common mutations have been identified in the human genome, thereby allowing high-density SNP microarrays. High-density SNP microarrays are powerful tools that enable

researchers to compare allele frequencies of these common SNPs in the genomes of diseased and healthy individuals. A typical GWAS study on human disease genetics includes a very large number of cases (affected with a particular disease) and controls (healthy participants) that are genotyped for a very large number of SNPs. To achieve the necessary power for detecting an association between an SNP marker and a disease phenotype, researchers should consider thousands of individuals, in which some GWAS studies include up to 200,000 samples (Ehret *et al.*, 2011).

Case-control studies are the most commonly used approach to perform genetic studies of complex phenotypes in GWAS. A candidate gene case-control study is conventional and commonly used method, in which allele and genotype frequencies are compared between cases and controls (Marian, 2012).

1.13 Environmental factors

The usage of alcohol, highly salted, pickled, fermented or smoked foods, exposure to metal and cement dust and cigarette smoking have all been linked to an increased risk of gastric cancer (Lochhead and El-Omar, 2008; Liu *et al.*, 2009). Many studies have found that high consumption of vitamin C can significantly reduce the risk of gastric cancer and thus playing the role of an antioxidant in preventing gastric cancer (Yuan *et al.*, 2004; Jenab *et al.*, 2006). On the contrary, consumption of nitrosamines, such as those commonly found in pickled vegetables have been found to induce gastric cancer *in vivo* (Liu *et al.*, 2009).

Smoking has been linked to gastric cancer. Studies done in a larger population have found that smoking is a risk factor for gastric cancer (Gonzalez *et*

al., 2003; Doll *et al.*, 2005). For those who quit smoking, the cancer risk can remain up to 14 years (Koizumi *et al.*, 2004). Salt has played important socioeconomic and cultural roles in the history of mankind. Salted preserved foods have a high salt concentration and contain food preservatives. Studies have found that, high salt intake predispose to gastric cancer and directly damage the gastric mucus (Wang *et al.*, 2009; Peleteiro *et al.*, 2011).

1.14 Summary of the study

The bacterium *Helicobacter pylori (H. pylori)* is indeed an extraordinary organism, able to colonize about 50% of the world population and causing disease in a significant proportion of them and yet has been able to remain hidden, only to have been discovered as recently as 1983 by Warren and Marshall, who were awarded with the Nobel Prize in Medicine 2005 (Marshall and Warren, 1984). This is more than 300 years after Anthony van Leeuwenhoek in 1675 first saw his "animalcules" under his microscope.

H. pylori is a Gram-negative, spiral shaped bacterium and one of the most common bacterial infections seen in humans. It is acquired early in life and persists for a very long period of time in the host. *H. pylori* is closely linked to chronic gastritis, peptic ulcer, gastric cancer and gastric mucosa-associated lymphoid tissue lymphoma (MALT) (Ben Mansour *et al.*, 2010). The bacterium has worldwide distribution and the frequency ranges from 25% in developed countries to more than 90% in developing areas, but not all infected individuals finally developed the disease (Ribeiro *et al.*, 2003). Infection with *H. pylori* has been shown to follow a geographic and socio-demographic distribution. Interestingly, the infection rate in

various populations does not parallel the incidence of morbidity and mortality caused by the infection (Yamaoka, 2009). Infection with this organism causes a major health burden upon health care systems.

The factors that lead to the development of the disease involve interaction between bacterial *H. pylori* related factors, host related factors and environmental factors (Izzotti *et al.,* 2009). A number of proteins, including *vacA, cagA, babA, dupA, SabA* and *iceA* have been suggested to play a role in the virulence of *H. pylori*, by increasing the severity of disease outcome (Lu *et al.,* 2005; Wu *et al.,* 2005; Aspholm *et al.,* 2006; Atherton, 2006). The clinical relevance of putative virulence-associated genes of *H. pylori* is still a matter of controversy. These genes may contribute to the exacerbation of mucosal damage. It has become apparent that not only the pathogen but also the host genetics and environmental factor play an important role in determining the clinical manifestation of *H. pylori* infections.

The cytotoxin-associated gene, *cagA*, a marker for the cag pathogenicity island (PAI), is present in many but not all *H. pylori* strains. *CagA* is deemed to be one of the most imperative virulence factors in the pathogenesis of *H. pylori*. The *cagA* gene is located at one end of the cag pathogenicity island (PAI) that codes a type IV secretion system (T4SS) and this secretion system is responsible for the translocation of *cagA* into host cells (Naito *et al.,* 2006). In Western countries, *cagA*-positive strains are reported to be linked with severe clinical outcomes, but in East Asian countries, it remains abstruse when trying to find this link because almost all *H. pylori* strains possess *cagA* (Yamaoka, 2010). After the delivery into the host cells, *cagA* protein is quickly tyrosine phosphorylated on specific tyrosine residues within repeating Glu-Pro-Ile-Tyr-Ala (EPIYA) motifs and interacts with various

target molecules. *CagA* protein is classified into two major types, Western and East Asian type, depending on the amino acid sequence surrounding the EPIYA motif (Azuma *et al.*, 2004). The EPIYA motif is found in four types of EPIYA segment, EPIYA-A, EPIYA-B, EPIYA-C and EPIYA-D (Higashi *et al.*, 2002a). Western- type strain possess EPIYA-A, EPIYA-B and EPIYA-C (Rudi *et al.*, 1998) while East Asian-type strains have EPIYA-A, EPIYA-B and EPIYA-D (Azuma *et al.*, 2004). Patients infected with East-Asian type *cagA* have an increased risk of peptic ulcer or gastric cancer when compared with those with Western-type *cagA* strains (Vilaichone *et al.*, 2004).

BabA is a blood-group antigen-binding adhesin encoded by the *babA2* gene, which has been shown to bind *H. pylori* to human Lewis b blood-group (Leb) antigens on gastric epithelial cells (Gerhard *et al.*, 1999). It has been reported that there is a significant relation between *babA2* positive genotypes and the occurrence of peptic ulcer diseases (Brandt *et al.*, 2005). The duodenal ulcer promoting gene (*dupA*) was initially described by Lu *et al.* in a study examining 14 vir gene homologues and their association with gastroduodenal disease especially with DU and hence the gene was named *dupA* (Lu *et al.*, 2005). *DupA* has been associated with an increased risk of DU and protection against GC in Japan and Korea (Lu *et al.*, 2005). The sialic acid-binding adhesin (*SabA*) has a capacity to bind to the glycosylated epithelial cells and this is considered to be important for *H. pylori* to cause constant infection and disease (Aspholm *et al.*, 2006; Odenbreit *et al.*, 2009). *SabA* was suggested to contribute to the chronic persistence of the infection (Mahdavi *et al.*, 2002; Aspholm *et al.*, 2006).

Host genetic polymorphisms affecting expression levels of important genes involved in pathogenicity have been demonstrated to influence susceptibility and severity of *H. pylori* infection. In general, genetic polymorphisms in pro-inflammatory genes tend to increase the risk of gastric cancer, as demonstrated for IL-1, a potent pro-inflammatory cytokine and the most prominent inhibitor of gastric acid secretion (Calam, 1999). Reduced acid secretion is linked to corpus-predominant colonization by *H. pylori,* which results in pangastritis formation of atrophic gastritis and thus an increased risk of gastric cancer and gastric ulcer disease (Zambon *et al.*, 2002; El-Omar *et al.*, 2003; Furuta *et al.*, 2004). Similar effects have been described for polymorphisms in other inflammation-associated genes, for example, the genes encoding tumor necrosis factor alpha (TNF-α) and IL-10 (Suzuki *et al.*, 2001).

It appears that patients with a pro-inflammatory genetic make-up based on a combination of markers from cytokine or chemokine genes (e.g., IL-1β, TNF-α, IL-10 and IL-8) and the innate immune response (e.g., TLR4 and MBL2), respond to *H. pylori* infection by creating a gastric environment that is chronically inflamed and with reduced acidity (Shanks and El-Omar, 2009).

Thousands of genes are being discovered for the first time by sequencing the genomes of model organisms, an exhilarating reminder that much of the natural world remains to be explored at the molecular level. DNA microarrays provide a natural vehicle for this exploration. DNA microarrays provide a simple and natural vehicle for exploring the genome in a way that is both systematic and comprehensive (Shalon *et al.*, 1996). Genomic variability can be present in many forms including Single nucleotide polymorphisms (SNP) and copy number variations (CNV). These

have been identified in several studies to be associated with complex diseases (Freeman *et al.*, 2006; McElroy *et al.*, 2009). Knowledge of the genomic variability present in diseases enable understanding the inherited basis of human variation in phenotypes, elucidating human physiology, evolution and disease.

This may be due to the presence of bacterial related genes or may be due to human genetic variability like single nucleotide polymorphism (SNPs) and copy number variations (CNVs) among the different ethnic groups in Malaysia. Therefore, this study will determine the genotypic frequencies of *cagA, dupA, babA2* and *sabA* of *H. pylori* genes in *H. pylori* infected patients and the host genetic factors and their roles in susceptibility to gastroduodenal disease.

1.15 Justification of the study

Approximately over 50% of the world populations are known to be infected with *H. pylori*. The percentage increases to 90% in developing countries (Ribeiro *et al.*, 2003). *H. pylori* infection causes gastric cancer, peptic ulcer and mucosa-associated lymphoid tissue (MALT) lymphoma. Although *H. pylori* infection leads to a wide range of gastroduodenal disease, only about 15% to 20% of people infected with *H. pylori* will develop ulcers (Wu *et al.*, 2008; Varbanova and Malfertheiner, 2011). The reason as to why only few people get gastric cancer or ulcer poses a major scientific challenge.

In developing countries, *H. pylori* infection management to prevent the occurrence of peptic ulcer and gastric cancer is highly expensive and thus causing

economic burden to the country. Therefore, knowing the contributing factors and its prevention is crucial particularly in low economic countries (Salih, 2009).

In Malaysia, three distinct ethnic groups predominate: Malays, Chinese and Indians. The prevalence of *H. pylori* infection is high and varies among the different ethnic groups. The Indians have been found to possess the highest prevalence of infection of about 68.9-75%, Chinese 45.0-60.6% and Malays 8-43.3% (Ramelah *et al.*, 2005; Tan *et al.*, 2005).

Interaction of several factors like environmental, bacterial virulence and host genetic are believed to determine the severity and final outcome of *H. pylori* infection. It is speculated that both host and bacterial factors play a role in disease development with differences in particular virulence determinants in circulating strains and host genetic make-up may play a role in susceptibility to *H. pylori* infection and severe gastroduodenal disease development. Thus, the aims of this study were to determine the distribution of bacterial virulence factors (*cagA*, *dupA*, *babA2* and *sabA*) and to investigate the host genetics susceptible factors to *H. pylori* infection in patient with gastroduodenal disease.

1.16 Hypothesis

The fundamental hypothesis of this study is that both host and bacterial factors play a role in disease development, the differences in the presence of specific virulence determinants in circulating strains among different ethnic groups and host genetic make-up may play a role in susceptibility to *H. pylori* infection and caused severe gastroduodenal disease development.

1.17 Objectives of the study

1.17.1 General Objective

The general aim of this study was to determine the distribution of bacterial virulence factors (*cagA*, *dupA*, *babA* and *sabA*) and specific human genetic factors (SNPs) role in determining the susceptibility of gastroduodenal diseases.

1.17.2 Specific Objectives

1. To determine the distribution of *cagA*, *dupA*, *babA2* and *sabA* of *H. pylori* virulence factors in *H. pylori* infected patients and to correlate with clinical outcome.
2. To determine the variability of *cagA* EPIYA motifs among *H. pylori* infected patients.
3. To determine the SNP profiles between patients infected with *H. pylori* and non-infected healthy controls.
4. To determine the genotype and phenotype association between SNP profiles of patients infected with *H. pylori*.

Chapter 2: Materials and Methods

2.1 Phase I: Detection of *H. pylori cagA, dupA, babA* and *sabA* and *cagA* EPIYA motifs

2.1.1 Materials

2.1.1.1 Bacterial strain

H. pylori ATCC 700392 (strain 26695) was used as a control strain in genotyping of *H. pylori* virulence genes.

2.1.1.2 Media preparations

2.1.1.2.1 Sterilization

The appropriate buffers, deionized water, micropipette tips, microcentrifuge tubes, PCR tubes and glasswares were sterilized by autoclaving at 121ºC, 15 psi for 15 min.

2.1.1.2.2 Columbia Horse Blood Agar

Twenty two grams of the Columbia blood agar base was suspended in 500 ml of purified water, mixed thoroughly, heated with frequent agitation and boiled for

one min to completely dissolve the powder. It was autoclaved at 121 ºC for 15 min. The Columbia agar base was cooled to 50 ºC and 35 ml of laked horse blood (SR0048) was added. Two mililitres of sterilized distilled water was mixed gently with *H. pylori* selective supplement, Dent (SR0147E) and the mixture was added to (Columbia agar base + 35 ml of laked horse blood (SR0048)). After mixing well it was dispensed into sterile petri dishes. The plates were stored at 4 ºC for three weeks in a plastic bag which maintains them in a moist environment.

2.1.1.2.3 Urease Agar

The ingredient was dissolved entirely in 95 ml of boiled distilled water. The medium was sterilized by autoclaving and cooled to 50 ^{o}C. After that, one ampoule of 40% sterile urea solution was added in the medium solution and mixed well. Lastly, the solution was distributed into 1.5 ml into each screw-capped test tube and stored at 4 ^{o}C.

2.1.1.2.4 Tryptone soya broth

Thirty grams of tryptone soya broth was added into 1 litre of distilled water, autoclaved at 121 ºC for 15 min and distributed into final containers.

2.1.1.2.5 Tryptone soya agar

Fourty grams was suspended in 1 litre of distilled water and boiled to dissolve completely. The mixture was autoclaved at 121 ºC for 15 min.

2.1.1.2.6 Brucella broth

Fourteen grams of Brucella powder, 0.5 gram of B-cyclodextrin and 75 ml glycerol were dissolved in 400 ml water and PH adjusted to 7.0 and the mixture was autoclaved at 121 ºC for 15 min. The mixture was then cooled at 46 ºC and 25 ml horse serum was added to the broth. Finally 500 µl of the mixture was divided into 1.5 ml test tube and kept at 4 ºC until used.

2.1.1.2.7 0.5 M Ethylenediaminetetraacetic Acid (EDTA)

EDTA solution was prepared by adding in 55.83 gram of EDTA in 200 ml of distilled water. The pH was adjusted to 8.0 by adding 3.0 M sodium hydroxide. Distilled water was added to make up to 300 ml.

2.1.1.2.8 10x Tris-Borate EDTA (TBE) Buffer

The buffer was prepared by dissolving 121.2 gram of Tris base, 63.8 gram of Boric acid and 0.75 gram of EDTA and was made up with distilled water to 1L. Then, the solution was sterilized using autoclave.

2.1.1.3 Agarose gel preparation

2.1.1.3.1 1% agarose gel

The gel was prepared by mixing 0.5 gram of agarose powder with 50 ml, 1X TBE (Tris Borate EDTA) buffer in a conical flask. The solution was mixed and

heated for 3-5 min in a microwave oven until the agarose dissolved completely. The solution was cooled by swirling the flask in a basin of water until 50 ◦C or until its warm enough to touch. One µl of gel red was added to the warm agarose and mixed well. The solution was poured onto assembled gel casting tray with appropriate gel comb without causing bubbles in the gel. The gel was left to solidify at room temperature for at least 30 min. When the gel has completely solidified, it was carefully removed.

2.1.1.3.2 1.5% agarose gel

The gel was prepared by mixing 1.2 gram of agarose powder with 80 ml, 1X TBE (Tris Borate EDTA) buffer in a conical flask. The solution was mixed and heated for 3-5 min in a microwave oven until the agarose dissolved completely. The solution was cooled by swirling the flask in a basin of water until 50 ◦C or until its warm enough to touch. One µl of gel red was added to the warm agarose and mixed well. The solution was poured onto assembled gel casting tray with appropriate gel comb without causing bubbles in the gel. The gel was left to solidify at room temperature for at least 30 min. When the gel has completely solidified, it was carefully removed.

2.1.1.4 Primers

2.1.1.4.1 Preparation of stock primers

The sources of all primers used in this study were supplied by Integrated DNA Technologies (IDT), (Singapore). The tubes containing lyophilized stock primers were centrifuge initially at 12,000 x g for 3 min and reconstituted with 100 µl nuclease free water. The tubes were vortexed until the pellet dissolve completely, then centrifuge and stored at -20 °C until further use. The stock solution concentration was calculated by dividing the amount of synthesized primers indicated on lyophilized stock tube with the volume of water used to reconstitute the primers (100 µl).

2.1.1.4.2 Preparation of working primers

A 20 pmol/ µl concentration of working solution was prepared from the stock solution by taking the appropriate volume of stock solution and adding it to a volume of nuclease-free water to make a final volume 200 µl. The solution was kept at -20 °C. The primers used in this study are indicated in (Table 3.7) The volume of stock solution and water was calculated by using the formula: $M_1V_1=M_1V_1$ where M_1= concentration of stock solution M_2= concentration of working solution V_1= volume of stock solution required V_2= final volume of working solution.

2.1.1.5 DNA extraction reagents

DNA extraction reagents were provided in the QIAamp DNA mini kit and blood Mini kit include:

2.1.1.5.1 ATL buffer

Animal tissue lysis (ATL) buffer used to induce lysis of cell tissues in order to release and expose the cell's DNA during the beginning stages of the extraction process. It is supplied in a total volume of 12 ml. The buffer was stored at room temperature. When a precipitation is formed in the buffer, it was incubated at 56 °C until fully dissolved. Buffer ATL is stable for 1 year when stored closed at room temperature.

2.1.1.5.2 Buffer AL (cell lysis solution)

Lysis buffer contains chaotropic salt and guanidinium chloride. Buffer AL is supplied in a total volume of 12 ml. The buffer was stored at room temperature. If a precipitation occur in buffer AL, it was removed by incubating the buffer at 70 °C until fully dissolved. Buffer AL is stable for 1 year when stored closed at room temperature.

2.1.1.5.3 Buffer AW1 and AW2 (column wash buffers)

Buffer AW1 and AW2 contains guanidinium chloride, which are used to wash and purify the extracted DNA from contaminants. Buffers AW1 and AW2 are

supplied as a concentrates in a total volume of 19 ml and 13 ml, respectively. They are diluted with pure ethanol (96–100%). Both of them are stable for 1 year when stored closed at room temperature

2.1.1.5.4 Proteinase K solution

Proteinase K is an enzyme that catalyzes the breakdown of cellular proteins by splitting them into smaller peptides and amino acids. The proteinase K solution can be stored at room temperature or at 2–8 °C. It is supplied in a total volume of 6 ml.

2.1.1.5.5 Buffer AE (elution buffer)

Buffer AE contains 0.5 mM Tris-HCL, pH 9.0. This solution elutes the DNA from the membrane and allows the collection of extracted DNA. Buffer AE is supplied in a total volume of 110 ml and is stored at room temperature (15-25 ^{o}C).

2.1.1.6 Equipments, chemicals, kits and list of consumables

The equipments, chemicals kits and list of consumables used in this study are indicated in Tables 2.1, 2.2, 2.3 and 2.4 respectively.

2.1.1.6.1 Equipment

Table 2.1 List of equipment used in this study with their sources

Name of equipment and instrument	Type of equipment and instrument	Name of company and origin
Anaerobic jar	Oxoid	England
Balance	Sortorius	Germany
Bijou bottle	Schott Duran	Germany
Bio-safety cabinet class II	NuAire, Inc	USA
Centrifuge machine	Eppendorf	Germany
Centrifuge mini spin	Eppendorf	Germany
Deep freeze(-20°C)	Medfrez	China
Freezer -80°C	Thermo Forma	USA
Heating block	Labnet Inc.,	USA
Ice maker	Scotsman	England
Incubator	Memmert	Germany
Vortex mixer	Heidolph instruments	Germany
Electrophoresis	NYX ECHNIK	Taiwan
Eppendorf centrifuge	Eppendorf	Germany
Gel image analyzer	G. Box	USA
Microwave oven	ELBA	Japan
Nanodrop device (ND-1000)	Thermo scientific	USA
PCR device	Eppendorf	Germany
pH meter	Eppendorf	USA
Pipette	Eppendorf	Germany
UV light transiluminator	Nurair	Germany
Vortex	Labnet Internationaline	USA
Water bath	Memmert	Germany
Autoclave	Nerima-KU	Japan
Cryobox	Helix biotech,	Malaysia
Ice maker	Scotsman,	England

2.1.1.6.2 Chemicals

Table 2.2 List of chemicals used in this study and their sources

Chemical compounds	Name of company
Absolute ethanol	Merck, Germany
Ethanol 95%	Merck, Germany
Columbia Agar base	Oxoid, England
Brucella broth base	Fluka, USA
Urea broth base	Oxoid, England
Culture media supplements	Oxoid, England
Agarose LE analytical grade	Promega, USA
Brain heart infusion agar	Oxoid, USA
Urea agar base	Oxoid, England
Tryptone soya broth	Oxoid, USA
Phenol red broth base	Remel, USA
Bromophenol blue	Thermo scientific, USA
Gram stain (Decoloriser)	Thermo scientific, USA
Gram stain (Crystal Voilet)	Thermo scientific, USA
Gram stain (Safranine)	Thermo scientific, USA
Gram stain (Iodine)	Thermo scientific, USA
Urea 40%	Oxoid, England
Horse serum	Oxoid, England
Sheep blood	Oxoid, England
Horse serum	Oxoid, England
Oxidase	Remel, USA
B-cyclodextrin	Sigma, USA
DNA Marker	Sigma, USA
Ethylenediamine tetra-acetic acid (EDTA)	Amresco, USA

Primers	Intergrated DNA Technologies, Singapore
Saline tablets	Oxoid, England
TBE buffer	1st Base, Singapore
Glycerol	Merck, Germany
AMPure magnetic bead	Bechman Coulter Genomics Danvers, USA
Gel red	Biotium, USA
Loading dye	Qiagen, Germany
Taq polymerase	Qiagen, Germany

2.1.1.6.3 Kits

Table 2.3 List of kits used in this study with their sources

Type of kit	Description and compounds	Name of company and origin
TopTaq Master Mix	1.25 units *TopTaq* DNA Polymerase 1 x PCR Buffer (contains 1.5 mM MgCl2) 200 µM of each dNTP	Qiagen (Germany)
QIAamp DNA Blood Kit	QIAamp mini spin columns, AL lysis Buffer, Proteinase K, AL buffer, AW1 washing buffer, AW2 washing buffer, AE (Elution buffer)	Qiagen (Germany)
QIAamp DNA Mini Kit	QIAamp mini spin columns, ATL lysis buffer , Proteinase K , AL buffer, AW1 washing buffer , AW2 washing buffer, AE (Elution buffer)	Qiagen (Germany)
DNA purification kit	QIAquik spin column, PB Binding buffer , PE Washing buffer , EB Elution buffer, pH Indicator,Collection tubes	Qiagen (Germany)

Table 2.4 List of consumables used in this study with their sources

Consumables	Name of the company and origin
Aluminium foil	Diamond, USA
Falcon tubes (15 ml)	Axygen, USA
Filter pipette tip (p10, p20, p100, p200, p500, p1000)	Axygen, USA
Latex free gloves (small & medium)	ScienceValley, Malaysia
Microcentrifuge tubes (0.5ml, 1.5ml, 2.0 ml)	Axygen, USA
Micropipette tips (10µl, 20µl, 100µl, 200µl, 1000µl)	Axygen, USA

2.2 Methods

The study was conducted among consenting adult patients with gastroduodenal disease who were referred to the Endoscopy Unit, Hospital Universiti Sains Malaysia and Hospital KualaLumpur in Malaysia. This study involves two phase, the first phase is a cross sectional and the second phase is case control study conducted from May 2012 to June 2014.

Phase I which is a cross-sectional phase of the study, three antral biopsies and stool sample were collected during upper endoscopy. In the invasive tests two biopsies samples were used to perform Rapid Urease test and culture. DNA was extracted from the third biopsy tissue for PCR detection of cagA, dupA, babA2 and SabA gens and cagA EPIYA motif. The stool sample was used for non-invasive tests (Atlas H. pylori antigen test).

The flow chart of the study is indicated in Figure 2.1. Biopsy tissue and stool sample were collected from dyspeptic patients who have fulfilled both inclusion and exclusion criteria at the time of the endoscopy. The biopsy tissue was used for culture, rapid urease test and PCR. *cagA*, *babA2*, *dupA*, *sabA* and *cagA* EPIYA motifs detection were identified by PCR. *H. pylori* was detected from stool sample by using *H. pylori* by Atlas *H. pylori* antigen test.

2.2.1.1 Study design

This current study is a cross-sectional study conducted from May 2012 to June 2014.

2.2.1.2 Study population and location

The study population were dyspeptic patients with upper gastrointestinal disease who attended Department of Medicine and Surgery, Endoscopy Unit, Hospital Universiti Sains Malaysia and Hospital Kuala Lumpur. The endoscopic diagnosis was grouped into four categories: gastritis, GU, DU and normal.

2.2.1.3 Sampling frame

The study was conducted among informed and consenting adult patients with gastroduodenal disease who were referred to the Endoscopy Unit, Hospital Universiti Sains Malaysia and Hospital Kuala Lumpur, Malaysia and fulfilled inclusion and exclusion criteria.

2.2.1.4 Sampling method

Due to lack of cases, nonprobability sampling method was applied in the current study.

2.2.1.5 Sample size determination

The sample size formula was calculated using two proportions for objectives one and two as shown below. The calculation of sample size was done using Power and Sample size (PS) Software (Dupont and Plummer Jr, 2010). Table 2.5 shows the sample size calculation for objective one and two. The highest sample size of 110 was selected for this study.

Table 2.5 Sample size calculation for first and second objectives

Genes	P_0	P_1	A	1-β	M	***n* per group**	**Total samples size and + 10% drop out**
cagA	0.54	0.80	0.05	0.8	1	50	110
babA2	0.61	0.88	0.05	0.8	1	40	88
dupA	0.65	0.93	0.05	0.8	1	32	70
sabA	0.09	0.34	0.05	0.8	1	41	90

The Power and Sample Size Software (PS) was used for calculations (Zheng *et al.*, 2006; Zhang *et al.*, 2008; Amjad *et al.*, 2010; Shao *et al.*, 2010).

2.2.1.5.1 *cagA* gene

1- Level of significance (α) is type I error, the probability of a two side test and this likelihood of wrongly rejecting the null hypothesis when the null hypothesis is true. In this study the type 1 error was set at 0.05 (Dupont and Plummer Jr, 2010).

2- Power (1-β). There is a probability that the test will correctly identify a significant difference or effect or association between contrasts in the samples, should one exist in the population. In this study the power was, 80%

3- Ƥ0 = The proportion of *cagA* gene among those without *H. pylori* infection (from literature)= 0.54

4- Ƥ1= The proportion of *cagA* gene among those infected with *H. pylori* (expert opinion) = 0.80

5- α = Level of significance = 0.05

6- 1-β = Power of study = 0.8

Based on calculations, the sample size required for *cagA* is 50 per group, with 10% addition of drop out, the minimum sample size required is 55 per group. Therefore the total subject required is 110 and this sample was selected because it gives the highest sample size.

2.2.1.5.2 *babA2* gene

1- Ῥ$_0$ = The proportion of *babA2* gene among those without *H. pylori* infection (from literature)= 0.61

2- Ῥ$_1$= The proportion of *babA2* gene among those infected with *H. pylori* (expert opinion) = 0.88

3- α = Level of significance = 0.05

4- 1-β = Power of study = 0.8

Based on calculations, the sample size required for *babA2* is 40 per group, with 10% addition of drop out, the minimum sample size required is 44 per group. Therefore the total subject required is 88.

2.2.1.5.3 *dupA* gene

1- Ῥ0 = The proportion of *dupA* gene among those without *H. pylori* infection (from literature)= 0.65

2- Ῥ1= The proportion of *dupA* gene among those infected with *H. pylori* (expert opinion) = 0.93

3- α = Level of significance = 0.05

4- 1-β = Power of study = 0.8

Based on calculations, the sample size required for *dupA* is 32 per group, with 10% addition of drop out, the minimum sample size required is 35 per group. Therefore the total subject required is 70.

2.2.1.5.4 *sabA* gene

1- Ῥ0 = The proportion of *sabA* gene among those without *H. pylori* infection (from literature)= 0.09

2- Ῥ1= The proportion of *sabA* gene among those infected with *H. pylori* (expert opinion) = 0.34

3- α = Level of significance = 0.05

4- 1-β = Power of study = 0.8

Based on calculations, the sample size required for *sabA* is 41 per group, with 10% addition of drop out, the minimum sample size required is 45 per group. Therefore the total subject required is 90.

2.2.1.6 Sample collection

Biopsy and stool samples were collected from the participant during endoscopy examination after obtaining written consent from each patient. A brief socio-demographic questionnaire was also filled from the patient. Three tissue biopsies were obtained from antrum. Rapid urease test was performed on one of the antral biopsies at the time of endoscopy. The other biopsy specimens for culture were placed in 1 ml of brucella broth. Another biopsy for detection of *cagA*, *dupA*, *babA* and *SabA* was also placed in 1 ml of brucella broth. If analysis was not done immediately the tissue was kept at −80°C until analysis.

2.2.1.7 Inclusion criteria and exclusion criteria

2.2.1.7.1 Inclusion criteria- Bacterial virulence genes

The inclusion criteria for bacterial virulence genes are

- 18 years and above
- Patients who are positive for *H. pylori*
- Patient with persistent abdominal pain, heartburn, acid regurgitation, sucking sensation, nausea and vomiting
- Discomfort over the preceding 3-month period
- Not been using nonstreroidal anti-inflammatory drugs (NSAID), antacids or any antibiotics within the previous two weeks
- Willing to participate in the study

2.2.1.7.2 Exclusion criteria

- Less than 18years
- Refused to give informed consent
- Patients with past history of *H. pylori* eradication therapy
- Patients taking antibiotics, H2-receptor blockers, bismuth or proton pump inhibitors in the preceding

2.2.1.8 Operational definitions

- Adult – a person > 18 years of age
- Invasive diagnosis- is the puncture of the skin or insertion of an instrument or foreign material into the body
- Noninvasive diagnosis- A medical procedure is strictly defined as non-invasive when no break in the skin is created and there is no contact with the mucosa, or skin break, or internal body cavity beyond a natural or artificial body orifice.
- Gastritis - Inflammation of the stomach lining that may result from infectionwith *H. pylori*, use of alcohol or tobacco, injury caused by certain medicines (such as aspirin), or autoimmune diseases
- Gastric ulcer- is an open sore in the lining of the stomach
- Duodenal ulcer- is a sore in the upper part of the small intestine (the duodenum)
- Peptic ulcer- is a distinct breach in the mucosal lining of the stomach (gastric ulcer) or the first part of the small intestine (duodenal ulcer)

2.2.1.9 Ethical approval

This study was approved by the Human Research Ethics Committee, Universiti Sains Malaysia (USMKK/PPP/JEPeM [247.3. (I7)], Kubang Kerian, Kelantan, Malaysia and National Medical Research Registry (NMRR-12-358-11418). Written informed consent was obtained from each patient prior to enrolling in the study.

2.2.1.10 Statistical analysis

The Statistical Package for Social Sciences (SPSS, IBM, and Chicago, USA) version 20.0) and Stata Version 11 (StataCorp, College Station, Texas, USA) were used for data entry and data analysis. All the data were double checked and prepared properly whilst being documented in order to detect missing data or errors.

The descriptive analysis was presented as mean and standard deviations (SD) for normally distributed numerical variables. Pearson Chi-square test was used for categorical variables; the data was presented as frequency (n) and percentage (%). Fisher exact test was used for categorical variables with 2x2 contingency tables, and Pearson Chi-square (χ^2) was selected for statistical comparison of each categorical data, if no less than 20% of cells had expected frequencies less than 5; Pearson Chi-square was applied. If equal or more than 20% of cells had expected frequencies less than 5, Fisher exact test was applied. Pearson Chi-square and Fisher exact test were used as appropriate for the comparison of individual virulence factors between ethnic groups and disease states. The Kappa coefficient (K) was calculated to assess the degree of agreement between the culture, RUT and Atlas *H. pylori* stool antigen test. The statistical significance was set at $p < 0.05$.

2.2.1.11 Rapid urease test (RUT)

Gastric antral biopsy were collected for rapid urease test. This test was performed with a homemade solution with 1 ml distilled water, one drop 1% phenol red, and 100 mg urea. One antral biopsy sample was placed in the solution immediately after endoscopy and maintained at room temperature. The test was

considered positive when the color changed from yellow to red within 24 hours (Pourakbari *et al.*, 2011).

2.2.1.12 Culture

2.2.1.12.1 Transport media

Gastric biopsy specimen for PCR and culture were placed in 500 µl of Brucella broth with 20% (v/v) glycerol and kept in −80 ˚C until processing.

2.2.1.12.2 Isolation of *H. pylori*

Gastric biopsy specimens were inoculated onto Columbia agar base (Oxoid) supplemented with 7% laked horse blood and *H. pylori* Dent's selective supplements (containing 5.0 mg/ml Vancomycin, 2.5 mg/ml, Trimethoprin, 2.5 mg/ml, Cefsulodin and 2.5 mg/ml, Amphotericin B). The plates were incubated for 5-7 days at 37 ◦C under microaerophilic conditions. Organisms were identified as *H. pylori* by Gram stain, oxidase, catalase and urease tests.

2.2.1.12.3 Gram staining

A colony of the microorganism was smeared on clean slide with a loopful of normal saline and allowed to dry before heat fixing it. The smear was flooded with crystal violet for about one min and removed by gently washing with tap water. The smear was then flooded with Gram iodine for one min and rinsed gently with tap

water. The smear was decolorized with alcohol until the smear is just colourless (10 seconds) and the slide then washed gently in tap water. Safranin as the counterstain was poured onto the smear for (30-60 seconds). The slide was washed with tap water and blotted and finally the slide was examined under the 100x objective lens. *H. pylori* was confirmed as Gram negative spiral rod.

2.2.1.12.4 Biochemical tests

2.2.1.12.4.1 Urease test

A few colonies of the test organism were inoculated in a Bijou bottle containing 3 ml of sterile urea broth. It was incubated for 30 min at 37 °C. The enzyme breaks down the urea to give ammonia and carbon dioxide. The medium, thus become alkaline when the indicator color changed from pale yellow to pink red, indicative of a positive reaction.

2.2.1.12.4.2 Catalase test

Two to three drops of the hydrogen peroxide solution was poured into a test tube. A single colony of the test organism was picked using a sterile wooden stick and immersed in the hydrogen peroxide. Catalase acts as a catalyst in the breakdown of hydrogen peroxide to oxygen and water. Bubbles of oxygen were released immediately indicating a positive reaction.

2.2.1.12.4.3 Oxidase test

Two to three drops of oxidase reagent (1% tetramethyl-p-phynelenediamine dihydrochloride) were dropped onto a piece of filter paper. A single colony was removed with a toothpick and smeared across the surface of the oxidase reagent impregnated filter paper. This test is to determine the presence of cytochrome oxidase and a dark purple color developed within 10 seconds would indicate a positive reaction.

2.2.1.13 Atlas *H. pylori* antigen test

Atlas *H. pylori* Antigen Test (Atlas medical, UK) is a rapid immunoassay using a monoclonal anti-*H. pylori* antibody on a strip for the detection of *H. pylori* infections in stool specimens. *H. pylori* antigen reacts with the conjugate-Pink Red latex particles sensibilized with anti-*H. pylori* monoclonal antibody coated to the membrane of the strip. The formed *H. pylori*-conjugate complex, which migrates upward the membrane by capillarity, binds to the specific antibody molecules fixed to the reaction zone.

The stool is collected in a clean container and the test done as soon as possible or stored at 2-8 ºC for a longer period of time. The test device and sample are put at room temperature (15-30 ºC) prior to testing. The test was performed according to the manufacturer's guidelines. By using the applicator stick of the provided sample diluent vial, a small portion of stool specimen is transferred into the sample diluent and mixed well by shaking gently. The tip of the vial was broken off and four drops were added to the sample well in the test device.

The test was read after 5 min of incubation. A positive test result is indicated by appearance of green band at (control line) and red band in the zone marked T (result line). The sample is considered negative when only one green band (control line) appears in the white central zone of the strip. If no colored bands appear or only one band appears in the T zone the result is regarded as invalid and if an inconclusive result is obtained, the test is repeated with a new strip.

2.2.1.14 Genomic DNA isolation from biopsy tissue

Genomic DNA was extracted from biopsy tissue by the use of QIAamp DNA Mini kit according to the manufacturer's instruction. The tissue was placed in 1.5 ml a microcentrifuge tube containing 180 µl of lysis buffer ATL. Twenty microlitre of Proteinase K solution was added, vortexed and then incubated at 56 °C overnight in a shaking water bath or until the tissue was completely lysed. The tissue lysate was centrifuged for about 1 min in order to mix the whole content. Two hundred microlitre of Buffer AL was added to the sample, mix by pulse-vortexing for 15 seconds, and incubated at 70 °C for 10 min. the 1.5 ml microcentrifuge tube was briefly centrifuged to remove drops from inside the lid. Two hundred microliter of ethanol (96-100%) was added to the sample and pulse– vortexed for 15 seconds and then centrifuged for about 1 min.

The mixture (including the precipitate) was applied to the QIAamp Spin Column in a 2 ml collection tube without wetting the rim. The cap was closed and centrifuged at 6000 x g for 1 min. The QIAamp Spin Column was placed in a clean 2 ml collection tube and the tube containing the filtrate was discarded. Five hundred microlitre of wash buffer (AW1) was added without wetting the rim. The cap was

closed and centrifuged at 6000 x g for 1 min and placed in a clean 2 ml collection tube. Again five hundred microlitre of wash buffer (AW2) was added without wetting the rim. The cap was closed and centrifuged at 20,000 x g for 3 min. To eliminate any possible buffer AW2 carryover, the QIAamp Spin Column was placed in a new 2 ml collection tube and the collection tube containing the filtrate was discarded, centrifuged at 20,000 x g for 1 min.

The QIAamp Spin Column was then placed in a clean 1.5 ml microcentrifuge tube and the collection tube containing the filtrate was discarded. The QIAamp Spin Column was opened and 50 µl elution buffer (AE) was added. It was incubated at room temperature for 5 min and then centrifuged at 6000 x g for 1 min. The QIAamp Spin Column was discarded and purified DNA was collected in 1.5 ml microcentrifuge tube and stored at – 20 °C until analysis.

2.2.1.14.1 Quantititation of DNA

The concentration of genomic DNA was measured by Nanodrop spectrophotometer ND-1000. DNA purity was determined by examining the A260/A280 ratio and the A260/A230 ratio. DNA was considered to be pure if the A260/A280 ratio is 1.8 – 2.0.

2.2.1.15 Detection of *H. pylori cagA, babA2, dupA* and *sabA* genes

For detecting the presence of the *cagA, babA2, dupA* and *sabA* in *H. pylori* genes, the following amplification and melting conditions were used.

2.2.1.15.1 Working solution

The PCR reaction mixtures were prepared by using TopTaq Master Mix Kit (Table 2.6) in a final volume of 25 µl containing 1.25 units *TopTaq* DNA *polymerase*, 1 X PCR buffer, 1.5 mM $MgCl_2$ and 200 µM of each dNTP, 0.2 µM of each primer, 9.5 µl of molecular grade water and 2.5 µl of DNA. The mixtures were placed in a conventional PCR thermocycler.

Table 2.6 Components of Top Taq master mix reaction

Component of master mix	**Volume/reaction**	**Final concentration**
Top taq PCR Master mix	12.5 µl	1.25 units *TopTaq* DNA *polymerase*, 1 X PCR buffer, 1.5 mM $MgCl_2$ and 200 µM of each dNTP
Forward primer (0.2 µM)	0.25 µl	0.2 µM
Reverse primer (0.2 µM)	0.25 µl	0.2 µM
Molecular grade water	9.5 µl	-
Tempelate DNA	2.5 µl	20ng/ µl
Total volume	**25 µl**	

2.2.1.15.2 PCR amplifications and condition for *cagA, babA2, dupA* and *sabA*

The PCR amplifications of *cagA*, *babA2*, *dupA* and *sabA* was done by using previously published primes as indicated in Table 2.7. The PCR conditions for *cagA* included an initial denaturation of target DNA at 94 °C for 1 min, followed by 35 cycles of denaturation at 94 °C for 1 min, primer annealing at 58 °C for 1 min, extension at 72 °C for 1 min and final extension at 72 °C for 15 min. As for *babA2* and *DupA* genes the PCR conditions are: initial denaturation 94 °C for 5 min, 35 cycles of denaturation at 94 °C for 45 s, primer annealing at 52 °C for 45 s, extension at 72 °C for 1 min and final extension of 72 °C for 5 min. The PCR condition for *sabA* involves an intial denaturation of 95 °C for 10 min, 35 cycles of denaturation at 95 °C for 20 s, annealing at 45 °C for 20 s, extension at 72 °C for 90 seconds and final extension of 72 °C for 5 min. PCR products were run on 1.5% agarose gels containing gel red in TBE buffer according to the manufacturer's instructions. The PCR conditions are shown in Table 2.8.

Table 2.7 Primers used for PCR amplification for *cagA*, *babA2*, *dupA* and *sabA*

Primer	**Primer sequence (5′-3′)**	**bp**	**References**
cagA-D008	GGTCAAAATGCGGTCATGG	297	(Domingo *et al.*, 1999; Sillakivi *et al.*, 2001)
cagA-R008	TTAGAATAATCAACAAACATCACGCCAT		
babA2	CCAAACGAAACAAAAAGCGT	271	(Sheu *et al.*, 2003)
	GCTTGTGTAAAAGCCGTCGT		
*dupA*1	CGTGATCAATATGGATGCTT	197	(Gomes *et al.*, 2008)
*dupA*2	TCTTTCTAGCTTGAGCGA		
sabA-F	CCGCTAGTGTCCAGGGTAAC	364	(Shao *et al.*, 2010)
sabA-R	CACCGCGTATTGCGTTGGGTA		

Table 2.8 PCR conditions for *cagA*, *babA2*, *dupA* and *sabA*

Genes	***cagA***		***babA2***		***dupA***		***sabA***	
	Temp	Time	Temp	Time	Temp	Time	Temp	Time
Initial denaturation	94 °C	1 min	94°C	5 min	94°C	5 min	95°C	10 min
Denaturation	94°C	1 min	94°C	45 s	94°C	45 s	95°C	20 s
Annealing	58°C	1 min	52°C	45 s	52°C	45 s	45°C	20 s
Extension	72°C	1 min	72°C	1 min	72°C	1 min	72°C	90 s
Final extension	72°C	15 min	72°C	5 min	72°C	5 min	72°C	5 min
Cycle	35		35		35		35	

2.2.1.15.3 Agarose gel electrophoresis

The PCR product was placed in the electrophoresis tank with TE buffer. Four microliter of DNA samples was mixed with 2 µl of 6X loading dye and loaded into the well. Hundred base pair molecular marker was put in the first and last lane. Once sample loading was done, the tank lid was closed and electrical connections were attached. Electrophoresis was started and the programme run for 1 hour at 80 V. The progress of separation was monitored by the migration of the loading dye. When the programme is finished, the power supply is switched off and the gel place on a UV transilluminator connected to a gel image capture system to visualize the bands.

2.2.1.16 Detection of cagA EPIYA motifs

For detecting the presence of the *cagA* EPIYA motifs in *H. pylori*, the following amplification and melting conditions were used.

2.2.1.16.1 Working solution

The PCR reaction mixtures were prepared by using *TopTaq* Master Mix Kit in a final volume of 25 µl containing 1.25 units *TopTaq* DNA polymerase, 1 X PCR buffer, 1.5 mM $MgCl_2$ and 200 µM of each dNTP, 0.2 µM of each primer, 9.5 µl of molecular grade water and 2.5 µl of DNA. The mixtures were placed in a PCR thermocycler.

2.2.1.16.2 PCR amplification and condition of *cagA* EPIYA motifs

PCR amplification of *cagA* was carried out using forward primer D008 and reverse primer R008 (Domingo *et al.*, 1999; Sillakivi *et al.*, 2001). PCR for detection of the EPIYA motifs was performed as previously described (Rudi *et al.*, 1998; Argent *et al.*, 2005) using the forward primer cag2 with each of the three reverse primers, *cagA*-P1C, *CagA*‑P2GC, *cagA*-P2TA and *cagA*-P3E. As *cagA*P3E could not distinguish between EPIYA-C and -D, we used primer *CagA*‑PD to amplify the EPIYA motif A, B, C, and D (Jones *et al.*, 2009). We confirmed the result by using two reverse primers, *cagA*-West and *cagA*-East, designed by Schmidt *et al* (Schmidt *et al.*, 2009b). The above primers were shown in Table 2.9.

The PCR conditions for *cagA* EPIYA motifs which is shown in Table 2.10 included an initial denaturation of target DNA at 94 °C for 1 min, followed by 35 cycles of denaturation at 94 °C for 1 min, primer annealing at 58 °C for 1 min, extension at 72 °C for 1 min and final extension at 72 °C for 15 min. PCR products were run on 1.5% agarose gels containing gel red stain in TBE buffer according to the manufacturer's instructions.

Table 2.9 Primers used for PCR amplification of *cagA* and *cagA* EPIYA motifs

Primer	**Primer sequence (5′-3′)**	**bp**	**references**
cagA- D008	GGTCAAAATGCGGTCATGG	297	(Domingo *et al.*, 1999; Sillakivi *et al.*, 2001)
cagA-R008	TTAGAATAATCAACAAACATCACGCCAT		
*Cag*2- F	GGAACCCTAGTCGGTAATG	550	(Rudi *et al.*, 1998)
*Cag*4- R	ATCTTTGAGCTTGTCTATCG	800	
*CagA*28- F	TTCTCAAAGGAGCAATTGGC		(Argent *et al.*, 2005)
CagA-P1C-R	GTCCTGCTTTCTTTTTATTAACTTKAGC	291	
(EPIYA -A)			
CagA-P2CG-R	TTTAGCAACTTGAGCGTAAATGGG	320	
(EPIYA -B)			
CagA-P2TA-R	TTTAGCAACTTGAGTATAAATGGG		
(EPIYA –B)			
CagA-P3E- R	ATCAATTGTAGCGTAAATGGG	501	
(EPIYA -C)			
CagA -PD- R	TTGATTTGCCTCATCAAAATC	400	(Jones *et al.*, 2009)
(EPIYA -D)			
*cagA*West-R	TTTCAAAGGGAAAGGTCCGCC	501	(Schmidt *et al.*, 2009b)
*cagA*East-R	AGAGGGAAGCCTGCTTGATT	495	

Table 2.10 PCR conditions for *cagA* EPIYA motifs

Step	**Temperature (°C)**	**Time**	**Cycle**
Initial denaturation	94	1 min	
Denaturation	94	1 min	
Annealing	58	1 min	35 ×
Elongation	72	1 min	
Final elongation	72	15 min	

2.2.1.16.2.1 Agarose gel electrophoresis

The agarose gel was placed into the electrophoresis tank with TBE buffer. Four microliter of DNA samples was mixed with 2 µl of 6X loading dye and loaded into the well. Hundred base pair molecular marker was usually put in first and last lane. Once sample loading is finished, the tank lid was closed and electrical connections were attached. Electrophoresis was started and the programme run for 1 hour at 80 V.

The progress of separation was monitored by the migration of the loading dye. When the programme is finished, the power supply is switched off and the gel place on a UV transilluminator connected to a gel image analyser G-Box system to capture the bands.

2.2.1.16.3 Sequencing of *cagA* Gene

2.2.1.16.3.1 Purification Protocol

One hundred µl of PB buffer was added to 20 µl of PCR products of the *cagA* gene in 1.5 ml eppendorf tube and mixed. To bind DNA, the *cagA* -PCR product sample was applied to QIAquik spin column and centrifuged at 20,000 x g for 1 min. The flow-through was discarded and the QIAquick column was returned into tube. In order to wash, 750 µl of PE buffer were added to the QIAquick column and centrifuged at 20,000 x g for 1 min. Then the flow-through was discarded and the QIAquick column was returned into tube. The column was centrifuged again at 20,000 x g for 1 min. After the centrifugation, the QIAquick column was placed in a

new 1.5 ml eppendorf tube. Lastly to elute DNA, 20 µl of EB elution buffer was added to the center of the QIAquick column membrane .The column was let to stand for 1 min, and then centrifuged at 20,000 x g for 1 min and the DNA sample was stored at -20 °C until use.

2.2.1.16.3.2 Sequencing of the 3' Variable Region of *cagA* Gene

Nucleotide sequencing of the purified products of the 3' variable region of *cagA* gene has been performed for 11 randomly selected strains including 7 from Gastritis, 2 from GU and 2 from DU patients. The purified PCR products were sent to 1st BASE Laboratory Sdn Bhd, Malaysia for sequencing in both directions. The name and sequence of primers (Cag2-F, Cag4-R) used in the sequencing are same as (Table 2.9). Applied Biosystems 3730XL DNA analyser was used for sequencing. Applied Biosystems 3730XL DNA analyser is the Gold Standard for high throughput genetic analysis. The sequencing result was received in BioEdit sequence alignment editor.

2.2.1.16.3.3 Translation to Amino Acids of *cagA* Variable Regions

Nucleotide sequences were translated into amino acid sequences using the Expasy translation tool which allows the translation of a nucleotide (DNA/RNA) sequence to a protein sequence.

2.2.1.16.3.4 Identification of *cagA* EPIYA Motif

In order to identify *cagA* EPIYA motif, all amino acid sequences of *cagA* variable regions were aligned together with amino acid sequences of previously published *cagA* gene sequence in GenBank http://www.ncbi.nlm.nih.gov/genbank.. Alignment of partial *CagA* peptide sequences was carried out using ClustalW built in BioEdit ver. 7.0.9 (http://www. BioEdit ver. 7.0.9/tools/clustalw/). Oligonucleotide screening by BLAST analysis was used to identify *H. pylori cagA* peptide sequences. Alignment of partial *cagA* peptide sequences was carried out using CLUSTAL W.

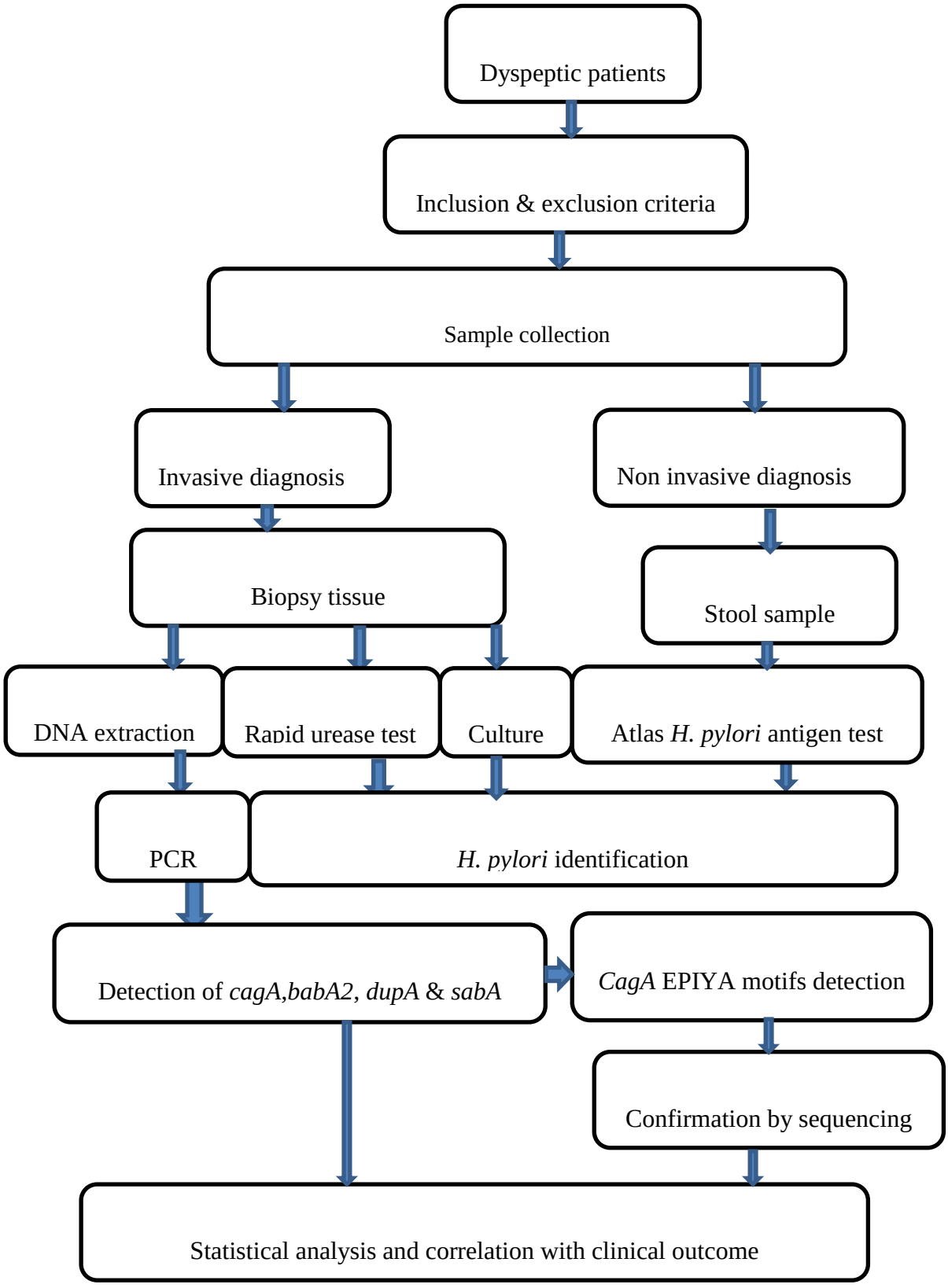

Figure 2.1 Flow chart of the study

2.3 Phase II: Genome wide association study

2.3.1 Materials

2.3.1.1 Equipment

The equipemnt used are indicated in Table 2.11

Table 2.11 List of equipment used in this study with their sources

Name of equipment and instrument	Name of the company and origin
Affymetrix Genome-Wide Human SNP Array 6.0	Affymetrix Santa Clara, CA, USA
GeneChip scanner 3000	Affymetrix Santa Clara, CA, USA
The GeneAmp® PCR System 9700	Thermo scientific, USA
Genechip fluidic station 450	Affymetrix Santa Clara, CA, USA
Genechip hybridization oven	Affymetrix Santa Clara, CA, USA
Genechip scanner	Affymetrix Santa Clara, CA, USA
NanoDrop	Thermo scientific, USA
Vortexer	Labnet Internationaline, USA
magnetic stand	Agencourt Bioscience, USA
Vortexer (with foam tube adaptor attached)	Eppendorf, Germany

2.3.1.2 Reagents

All the reagents described below are products of (Affymetrix, USA).

2.3.1.2.1 *Nsp* and *Sty* Restriction Enzyme Digest

This kit includes BSA (100X; 10 mg/mL), NE Buffer 2 (10X), NE Buffer 3 (10X), *Nsp*I (10 U/µl; NEB), *Sty*I (10 U/µl; NEB) and AccuGENE Water.

2.3.1.2.2 *Nsp* and *Sty* Ligation

Nsp and *Sty* ligation reagents are T4 DNA Ligase (400 U/µl; NEB), T4 DNA Ligase Buffer (10X), Adaptor, *Nsp* (50 µM), Adaptor, *Sty* (50 µM) and AccuGENE water.

2.3.1.2.3 *Nsp* and *Sty* PCR

The PCR kit contains AccuGENE water, molecular biology-grade, PCR Primer 002 (100 µM), dNTPs (2.5 mM each), GC-Melt (5M), *TITANIUM*™ *Taq* DNA Polymerase (50X), *TITANIUM*™ *Taq* PCR Buffer (10X).

2.3.1.2.4 PCR Product Purification

Reagents needed for purification includes Agencourt AMPure® magnetic beads, 75% EtOH (ethanol diluted to 75% using AccuGENE water) and Elution Buffer (Buffer EB).

2.3.1.2.5 Quantitation

AccuGENE water.

2.3.1.2.6 Fragmentation

Fragmentation reagents are , Fragmentation Buffer (10X), Fragmentation Reagent (enzyme; DNase I) and AccuGENE water.

2.3.1.2.7 Labeling

For labelling the reagents are DNA Labeling Reagent (30 mM), Terminal Deoxynucleotidyl Transferase (TdT; 30 U/µl) and Terminal Deoxynucleotidyl Transferase Buffer (TdT Buffer; 5X).

2.3.1.2.8 Target Hybridization

During hybridization the reagents used includes Hyp buffer part 1, 2, 3 and 4 and OCR.

2.3.1.3 Agarose gel preparation

2.3.1.3.1 2% agarose gel

The gel was prepared by mixing 1gram of agarose powder with 50 ml, 1X TBE (Tris Borate EDTA) buffer in a conical flask. The solution was mixed and

heated for 3-5 min in a microwave oven until the agarose dissolved completely. The solution was cooled by swirling the flask in a basin of water until 50 ˚C or until its warm enough to touch. 1 µl of gel red was added to the warm agarose and mixed well. The solution was poured onto assembled gel casting tray with appropriate gel comb without causing bubbles in the gel. The gel was left to solidify at room temperature for at least 30 min. When the gel has completely solidified, it was carefully removed. This was used after PCR step in microarray process.

2.3.1.3.2 4% agarose gel

The gel was prepared by mixing 2 gram of agarose powder with 50 ml, 1X TBE (Tris Borate EDTA) buffer in a conical flask. The solution was mixed and heated for 3-5 min in a microwave oven until the agarose dissolved completely. The solution was cooled by swirling the flask in a basin of water until 50 ◦C or until its warm enough to touch. 1 µl of gel red was added to the warm agarose and mixed well. The solution was poured onto assembled gel casting tray with appropriate gel comb without causing bubbles in the gel. The gel was left to solidify at room temperature for at least 30 min. When the gel has completely solidified, it was carefully removed. This was used after fragmentation step in microarray process.

2.4 Methods

At the time of endoscopic examination 3 ml of blood sample were collected into EDTA by qualified nurse from the patients after obtaining written consent from each patient. Genomic DNA was extracted from blood sample by the use of QIAamp DNA blood Mini kit according to the manufacturer's instruction and used for SNP 6.0 microarray analysis. A brief socio-demographic questionnaire was also filled from the patient.

Genotyping was performed using the Affymetrix SNP 6.0 Array platform according to the manufacturer's instructions. Briefly, 250 ng of genomic DNA was digested with restriction enzyme and ligated with ligase enzyme. The ligated products were then PCR amplified. Amplicons were electrophoresed, purified and quantified to ensure that the samples passed quality control (QC) measures before further experiment. The products were then fragmented, hybridized onto the Affymetrix SNP 6.0 chips and stained. Chips were scanned and raw data was generated using Affymetrix Genotyping Console Software (GTC) version 3.0.2. (Figure 2.6).

2.4.1 Study design

This is a case control study conducted from May 2012 to June 2014.

2.4.2 Study population and location

The study population were dyspeptic patients with upper gastrointestinal disease who attended Department of Medicine and Surgery, Endoscopy Unit, Hospital Universiti Sains Malaysia and Hospital Kuala Lumpur. The endoscopic diagnosis was grouped into four categories: gastritis, gastric ulcer (GU), duodenal ulcer (DU) and normal.

2.4.3 Sampling frame

The study was conducted among informed and consenting adult patients with gastro duodenal disease who were referred to the Endoscopy Unit, Hospital Universiti Sains Malaysia and Hospital Kuala Lumpur, Malaysia and fulfilled inclusion and exclusion criteria.

2.4.4 Sampling method

Due to lack of cases, nonprobability sampling method was applied in the current study.

2.4.5 Sample size determination

Due to high cost of microarray chips and considering the study budget, a total of 80 samples including both case and control were selected and proceed for SNP 6 genotype analysis.

2.4.6 Sample collection

Three mililitre of blood was collected into EDTA by qualified nurse from the patients. DNA was extracted and samples stored at −80°C until microarray analysis.

2.4.7 Inclusion and exclusion criteria

2.4.7.1 Inclusion criteria SNP analysis cases

- 18 years and above
- Patients who are positive for *H. pylori*
- Patient with persistent abdominal pain, heartburn, acid regurgitation, sucking sensation, nausea and vomiting
- Discomfort over the preceding 3-month period
- Patient's who have not been using nonstreroidal anti-inflammatory drugs (NSAID), antacids or any antibiotics within the previous two weeks
- Three generation ethnic group
- Willing to participate in the study

2.4.7.2 Exclusion criteria SNP analysis cases

- Less than 18 years
- Patients who are negative for *H. pylori*
- Below three generation ethnic group
- Not willing to participate in the study

2.4.7.3 Inclusion criteria SNP analysis control

- *H. pylori* negative
- Male or female above 18 years
- Three generation ethnic group
- Patient with persistent abdominal pain, heartburn, acid regurgitation, sucking sensation, nausea and vomiting

2.4.7.4 Exclusion criteria SNP analysis control

- Male or female below 18 years
- Below three generation ethnic group
- Not able to give informed consent.

2.4.8 Ethical endorsement

This study was approved by the Human Research Ethics Committee, Universiti Sains Malaysia (USMKK/PPP/JEPeM [247.3.(I7)], Kubang Kerian, Kelantan, Malaysia and National Medical Research Registry (NMRR-12-358-11418). Written informed consent was obtained from each patient prior to enrolling in the study.

2.4.9 Bioinformatics analysis

2.4.9.1 Affymetrix GeneChip® Command Console software (AGCC)

AGCC provides broad data collection and analysis tool for the Affymetrix GeneChip Platform. The suite offers automated data collection and instrument control for the GeneChip® Fluidics Station 450 and the GeneChip Scanner 3000. Using advanced scientific algorithms, the suite provides several different analysis applications. Analysis options include conversion of intensity data into expression results, allele detection, singular nucleotide polymorphism detection, and nucleotide analysis.

2.4.9.2 Affymetrix® Genotyping Console™ software (GTC)

The Affymetrix® Genotyping Console™ software (GTC) is application to calculate genotype calls and to generate copy number. Genotyping console uses cell intensity (CEL) files and genotyping analysis result (CHP) files to create copy number analysis from affymetric data.

2.4.9.3 Plink (version 1.07) software

PLINK (version 1.07) is a free, open-source whole genome association analysis toolset, designed to perform a range of basic, large-scale analyses in a computationally efficient manner. Before association studies was done by plink, the filtering was done by SNP filtering parameters; those having a minor allele

frequency (MAF) > 0.01, those deviating from Hardy-Weinberg equilibrium HWE p-value > 0.001 and SNP call rate > 95%. After the filtering, association studies for SNPs that pass filtering process were done by chi-squared (x^2) test. This test utilizes statistical measures such as minor allele frequency, major allele frequency and P-value that are suited to find genotype-phenotype association. The Manhattan was plotted to visualize GWAS results. Manhattan plot displays a plot of the $-\log_{10}$ (P-value) of the association statistic on the y-axis versus the chromosomal position of the SNP on the x-axis. The SNPs with the smallest p values shows higher dots in the Manhattan plot, indicating the stronger the genetic association.

2.4.10 Genomic DNA isolation from blood sample

Genomic DNA was extracted from blood sample by the use of QIAamp DNA blood Mini kit according to the manufacturer's instruction. Two hundred microlitre of blood sample was added into 1.5 ml microcentrifuge tube containing 20 µl QIAGEN Protease (or proteinase K). Two hundred microlitre of Buffer AL was also added to the mixture and vortexed for 15 seconds, incubated at 56 °C for 10 min and briefly centrifuged to remove drops from the inside of the lid.

Two hundred microlitre of ethanol (96–100%) was then added to the sample, mixed again by pulse-vortexing for 15 seconds and briefly centrifuged to remove drops from the inside of the lid. The mixture was transferred to the QIAamp Mini spin column (in a 2 ml collection tube) without wetting the rim, centrifuged at 6000 x g for 1 min and the QIAamp Mini spin column was placed in a clean 2 ml collection tube after discarding the tube containing the filtrate.

The QIAamp Mini spin column was opened and added 500 µl Buffer AW1 without wetting the rim, centrifuged at 6000 x g for 1 min and the QIAamp Mini spin column was placed in a clean 2 ml collection tube after discarding the collection tube containing the filtrate. Then 500 µl Buffer AW2 was added without wetting the rim, centrifuged at 20,000 x g for 3 min. To eliminate any possible buffer AW2 carryover, the QIAamp Spin Column was placed in a new 2 ml collection tube and the collection tube containing the filtrate was discarded, Centrifuged at 20,000 x g for 1 min.

After discarding the collection tube containing the filtrate, the QIAamp Mini spin column was placed in a clean 1.5 ml microcentrifuge tube and 50µl of reduced EDTA TE buffer was added into it. It was incubated at room temperature for 5 min and then centrifuged at 6000 x g for 1 min. Finally, the QIAamp Spin Column was discarded and purified DNA collected in 1.5 ml microcentrifuge tube and stored at -20°C until analysis.

2.4.10.1 Quantititation of DNA

The concentration of genomic DNA was measured by Nanodrop spectrophotometer ND-1000. DNA purity was determined by examining the A260/A280 ratio and the A260/A230 ratio. DNA was considered to be pure if the A260/A280 ratio is 1.8 – 2.0.

2.4.11 Sample preparation

The genomic DNA and positive control were thawed on ice. Once thawed, they were placed in the cooling chamber on ice. The concentration in the form of OD measurement of each sample was taken using Nanodrop spectrophotometer. The genomic DNA was thoroughly mixed by vortexing at high speed for 3 seconds and then spun down for 30 seconds and each sample diluted to 50 ng/µl using reduced EDTA TE buffer (10 mM Tris, 0.1 mM EDTA, pH 8.0).

In order to confirm concentration and purity, 1 µl of DNA with 1 µl Syber green and 1 µl loading dye is run on gel electrophoresis (1% TBE agarose gel) for 30 min at 90 Voltage. A good genomic DNA will produce bands without smear. A 96-well plate was marked in different colours (a blue marker was used for *Nsp* (N) and a red marker for *Sty* (S) and placed on the cooling chamber.

Two 5 µl aliquots of the first sample were transferred to the respective wells. The remaining samples were transerred in the same manner into the appropriate wells. The positive control was vortexed for 3 seconds then spun down for 30 seconds. Five microliter of the control sample was transferred to the wells and 5 ul of water which served as negative control was transferred into wells. The plate was tightly sealed. If the next step was not continued immediately, it was stored in -20°C.

2.4.11.1 *Nsp* and *Sty* restriction enzyme digestion

In this stage, 5 µl of genomic DNA (50ng/µl) is added into the wells of 96 well plate after vortexing. The reagents to be used for both *Sty* and *Nsp* restriction

enzyme digestion is thawed on ice before preparing digestion master mix of 14.75 µl in 1.5 ml Eppendorf tube (Table 2.12). The master mix is then added into the wells containing genomic DNA.

The plate is then sealed tightly by using adhesive film. The center of the plate is vortexed at high speed for 3 seconds and spun down at 2000 x g for 30 seconds. The plate is then loaded onto the pre- heated thermal cycler and the Cyto digest program was run. After the program is finished, the plate was removed and spun down at 2000 x g for 30 seconds.

The restriction enzyme digestion for thermal cycler includes, 37 °C for 120 min and 66 °C for 20 min as indicated in Table 2.13. after completion the PCR product is held at 4 °C. Then the ligation stage is commenced or samples were stored at -20°C

Table 2.12 Preparation of *Sty* I and *Nsp* 1 Digestion Master Mix (Genome-Wide SNP 6.0 Manual)

Reagent	**1 sample *Nsp***	**1 sample *Sty***	**Final concentration in sample**
Water, AccuGENE	11.55 µl	11.55 µl	
NE buffer 2 (Nsp MM only) 10X	2 µl	2 µl	1X
NE buffer 2 (Sty MM only) 10X	2 µl	2 µl	1X
BSA (100X; 10 mg/mL)	0.2 µl	0.2 µl	1X
Nsp 1 (10 U/µl)	1 µl	___	0.7 U /µl
Sty I (10 U/µl)	___	1 µl	0.7 U /µl
Total volume	14.75 µl	14.75 µl	

Table 2.13 Thermal cycler program for *Sty* and *Nsp* enzyme (Genome-Wide SNP 6.0 Manual)

Temperature	Time
37°C	120 min
65°C	20 min
4°C	Hold

2.4.11.2 *Nsp* and *Sty* ligation

The reagents required for ligation was thawed on ice. The master mix for ligation was prepared in 1.5 ml Eppendorf tube (Table 2.14). after preparing the master mix, 5.25 µl of both *Nsp* and *Sty* ligation master mix was added into the wells containing *Nsp* and *Sty* digested DNA.

The plate was then sealed tightly by using adhesive film. The center of the plate was vortexed at high speed for 3 seconds and spun down at 2000 x g for 30 seconds. The plate was then loaded onto the pre- heated thermal cycler and the Cyto ligate thermal cycler program was run at 16 °C for 180 min and 70 °C for 20 min. After completion the PCR product was held at 4 °C (Table 2.15). After the program is finished, the plate was removed and spun down at 2000 x g for 30 seconds.

Prior to PCR, the 25 µl of both *Nsp* and *Sty* ligated DNA was diluted with 75 µl molecular biology grade water. The plate was sealed tightly with adhesive film, vortex at high speed for 3 seconds and spun at 2000 x g for 30 seconds.

Table 2.14 Preparation of *Nsp* and *Sty* I Ligation Master Mix (Genome-Wide SNP 6.0 Manual)

Reagent	**1 sample *Nsp***	**1 sample *Sty***	**Final concentration in sample**
T4 DNA Ligase Buffer (10X)	2.5 µl	2.5 µl	4.5X
Nsp 1adaptor (50 µM)	0.75 µl	___	7 µM
Sty 1 adaptor (50 µM)	___	0.75 µl	7 µM
T4 DNA Ligase (400 U/µl)	2 µl	2 µl	150U/µl
Total	**5.25 µl**	**5.25 µl**	

Table 2.15 Thermal Cycler Program for ligation (Genome-Wide SNP 6.0 Manual)

Temperature	**Time**
16°C	180 min
70°C	20 min
4°C	Hold

2.4.11.3 *Nsp* and *Sty* PCR

Ten microlitre of each *Nsp* ligated sample was transferred to the corresponding four wells of the PCR plate and 10 µl of each *Sty* ligated sample was also transferred to the corresponding three wells of the PCR plate. The remaining ligated *Nsp* and *Sty* samples in the plate were sealed, labeled and stored at –20 °C.

PCR master mix (Table 2.16) was prepared in a 50 ml tube. 90 µl of *Nsp* and *Sty* PCR master mix was aliquoted into a fresh wells containing 10 µl ligated DNA sample, thus giving a total volume of 100 µl.

The plate was covered with adhesive film, vortexed and spun at 2000 x g for 30 seconds. The plate was then placed in a pre-heated thermal cycler and Cyto PCR program was run (Table 2.17). The plate was then removed from the thermal cycler and spun down at 2000 x g for 30 seconds and placed in a cooling chamber on ice.

The PCR amplification products was then checked by running 3 µl of each PCR product mixed with 3 µl of 2X gel loading dye on 2% agarose gel at 120V for 40 min. The PCR product distribution was verified between ~250 bp to 1100 bp (Figure 3.2). If not proceeding directly to the next stage, the plate was sealed with PCR product and stored at –20 °C.

Table 2.16 Preparation of Master Mix for PCR (Genome-Wide SNP 6.0 Manual)

Reagent	**1 sample *Nsp***	**1 sample *Sty***	**Final concentration**
Water, AccuGENE	39.5 µl	39.5 µl	
TITANIUM *Taq* PCR Buffer (10X)	10 µl	10 µl	1X
GC-Melt (5M)	20 µl	20 µl	1M
dNTP (2.5 mM each)	14 µl	14 µl	380 µM
PCR Primer 002 (100 µM)	4.5 µl	4.5 µl	5 µM
TITANIUM *Taq* DNA Polymerase (50X)	2 µl	2 µl	1X
Total	**90 µl**	**90 µl**	

Table 2.17 Thermal Cycler Program for the GeneAmp® PCR System 9700 (Genome-Wide SNP 6.0 Manual)

Temperature	**Time**
94°C	3 min
94°C	30 sec
60	45 sec
68	15 sec
68	7 min
4°C	Hold

2.4.11.4 PCR Product Purification using AMPure XP Beads

If frozen, the PCR products were thawed in a plate holder on the bench top to room temperature. The PCR product was vortexed at high speed for 3 seconds and each plate was spun down at 2000 x g for 30 seconds. Seven aliquots of each sample (*Nsp* and *Sty* PCR products) were transferred to the marked 2 ml microcentrifuge tube (Figure 2.2) making a total of 694 µl per well. The PCR plate was then examined to ensure that the total volume in each well has been transferred and pooled.

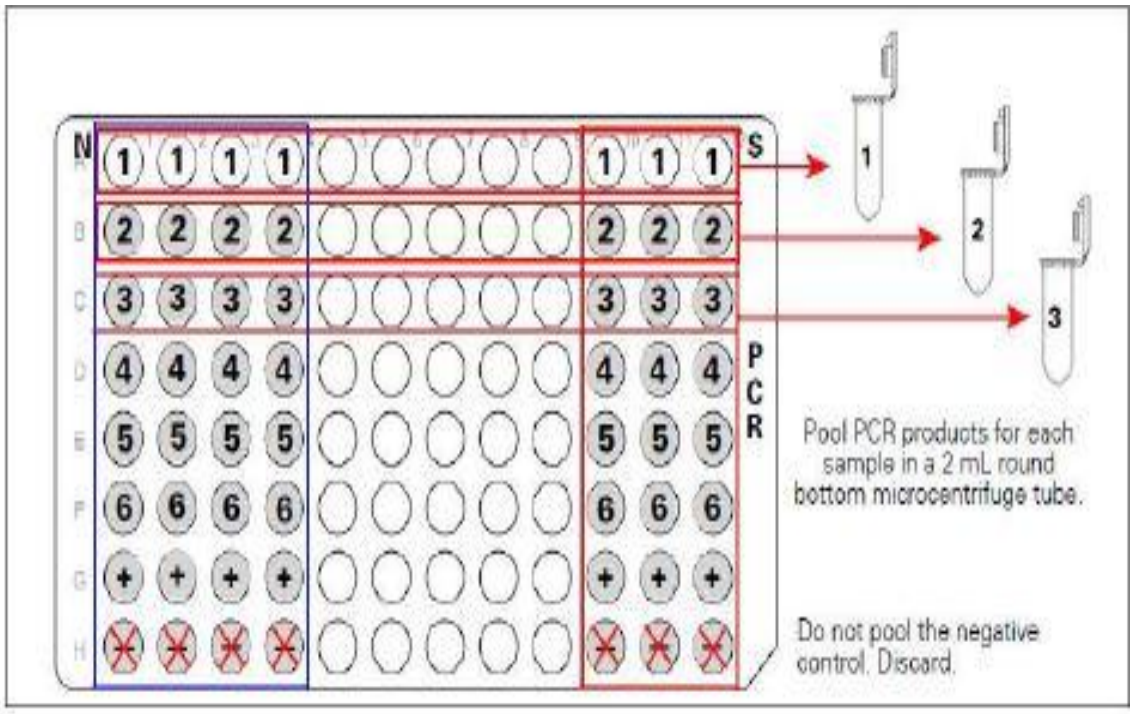

Figure 2.2 Pooling PCR products

The magnetic bead stock were mixed very well by vigorously shaking the bottle until the solution appears homogenous. One ml of magnetic beads was added to each well of pooled product. Each tube was securely capped and mixed well by inverting 10 times. It was then incubated at room temperature for 10 min to allow the DNA binds to the magnetic beads. The tubes were then loaded with cap hinge facing out onto the microcentrifuge and spun for 3 min at maximum speed (16,100 rcf).The tubes were then placed on the magnetic stand and the supernatant was removed and discarded without disturbing the bead pellet.

One thousand and five hundred microliters of 75% EtOH were added to each tube. The tubes were capped and loaded into the foam tube adaptor and vortexed at 75% power for 2 min. the tube was centrifuged for 3 min at maximum speed (hinges facing out; 16,100 rcf) and later placed back on magnetic stand. The supernatant was removed and discarded without disturbing the bead pellet. Then the tubes were spun at maximum speed for 30 seconds (hinges facing out; 16,100 rcf) and placed back on the magnetic stand. Remaining drops of EtOH removed from the bottom of each tube and the tubes uncapped and left at room temperature for 15 min in order for EtOH to evaporate completely. Fifty microlitres of Elution Buffer was added to each tubes and they were then capped and loaded into the foam tube adaptor. The tubes were then vortexed at 75% power for 10 min to resuspend the beads fully. If the beads were not fully resuspended, the tube was vortexed at 75% power for an additional 2 min. The tubes were centrifuged for 5 min at maximum speed (hinges facing out; 16,100 rcf) and placed on magnetic stand for 5 min until all beads were pulled to the side. Finally 47 µl of purified sample was transferred to a fresh well plate. The concentration and purity of purified PCR products are determined.

2.4.11.5 Quantification

In this stage, 198 µl of water is aliquoted into each well and then 2 µl of each purified PCR product of the purified sample plate was transferred to the corresponding wells of the optical. The plate was sealed, vortexed and spun down. The optical density (OD) of the PCR products was determined by spectrophotometer. The DNA yield should fall within the range 0f 4.5 to 7.0 µg/µl. The OD260/OD280 ration should be between 1.8 to 2.0. If not proceeding immediately to the next step, the plate was sealed and stored at –20 °C.

2.4.11.6 Fragmentation

Five microlitre of 10X Fragmentation Buffer was aliquoted into each sample, vortexed and placed back on ice. Then 5 µl of fragmentation master mix was added to the sample, giving a final volume of 55 µl. The plate was then sealed tightly, vortexed at high speed for 3 seconds then spun down for 30 seconds. The samples were immediately loaded onto the pre-heated block of the thermal cycler and the Cyto Fragment program was run (Table 2.18).

The fragmentation product was checked by running 2 µl of each fragmentation product mixed with 4 µl 6X loading dye on 4% of agarose gel at 120 V for 30 min. the fragmentation product should be < 180 bp in size.

Table 2.18 Thermal Cycler Program for fragmentation (Genome-Wide SNP 6.0 Manual)

Temperature	**Time**
37ºC	35 min
95ºC	15 min
4ºC	Hold

2.4.11.7 Labeling

Labeling should be done immediately after fragmentation. The labeling master mix (Table 2.19) was prepared in 1.5 ml eppendorf tube and 19.5 µl of the labeling master mix was aliquoted into the wells containing 53 µl fragmented DNA sample, thus giving a total of 73 µl. The plate was covered with adhesive seals, vortexed and spun at 2000 x g for 30 seconds and placed in a thermal cycler and labeling program was run as shown in (Table 2.20). Once labelling was completed, the product was stored at -20 ºC or proceeded with hybridization.

Table 2.19 Preparation of Master Mix for Labeling (Genome-Wide SNP 6.0 Manual)

Reagent	**1 sample**	**Final concentration in sample**
TdT Buffer (5X)	14 µl	3.6 X
DNA Labeling Reagent (30 mM)	2 µl	3 mM
TdT enzyme (30 U/µl)	3.5 µl	5.3 U/µl
Total	**19.5 µl**	

Table 2.20 Thermal Cycler Program for labeling (Genome-Wide SNP 6.0 Manual)

Temperature	**Time**
37ºC	4 hours
96ºC	15 min
4ºC	Hold

2.4.11.8 Hybridization

Hybridization master mix was prepared in 1.5 ml tube according to (Table 2.21) and 190 µl of master mix was added to each sample. The plate was sealed, vortexed, spun and cyto hybridization program was run on thermal cycler as indicated in (Table 2.22).

The arrays were allowed to equilibrate at room temperature prior to use for 10 to 15 min and the front or back of each array was marked with a designation that will identify which sample was loaded onto each array. The hybridization oven was preheated for 1 hour at 50 ºC with the rotation turned on and the rpm was set to 60. The lid of the thermal cycler was opened when the temperature reached 49 °C. While the sample was on the thermal cycler, 200 µl of each sample was removed and immediately injected into each array. Any excess fluid was cleaned from around the septa and tough-spots was applied to the septa and pressed firmly. Finally the arrays were loaded into the hybridization oven four at a time and run for 16 to 18 hours at 50 ºC and 60 rpm. The oven was ensured balanced and the trays were rotating at 60 rpm. After hybridization the arrays were removed and filled with array holding buffer if there is any bubble.

Table 2.21 Preparation of master mix for Hybridization (Genome-Wide SNP 6.0 Manual)

Reagent	**Per sample**
Hyp buffer part 1	165 µl
Hyp buffer part 2	15 µl
Hyp buffer part 3	7 µl
Hyp buffer part 4	1 µl
OCR	2 µl
Total	**190 µl**

Table 2.22 Thermal Cycler Program for Hybridization (Genome-Wide SNP 6.0 Manual)

Temperature	**Time**
95ºC	10 min
49ºC	hold

2.4.11.9 Washing and staining of the arrays

The fluidics station 450 (Figure 2.3) which does automatic staining and washing was used for this procedure. The fluidics station is controlled by the GeneChip® command console® software AGCC, version 1 as indicated in array plate processing workflow (Figure 2.4). Prior to washing and scanning, the sample were registered as new sample in the AGCC software. The sample number and array type are registered in the database by using AGCC portal. Priming of the fluidics station was done to ensure all the lines of the fluid stations were filled with appropriate buffer. Priming was carried out by using AGCC fluidics station control. To prime the fluidics station, the fluidics station was turned on and primed by selecting protocol prime_450 for each module.

The arrays were removed from the oven after 16 to 18 hours of hybridization. The hybridization cocktail from each array was extracted and transferred to the corresponding well of a 96-well plate. The arrays was stored on ice during the procedure or at –80 °C for long- term storage. Each array was filled completely with 270 µl of 1X Array Holding Buffer. The arrays were allowed to equilibrate to room temperature before washing and staining.

Affymetrix staining procedures uses three reagents, these are: Streptavidin Phycoerithrin stain (SAPE), anti-streptavidin biotinylated antibody stains and array holding buffer. The appropriate probe array and the three tubes containing the stains are inserted into the fluidics station. Buffer A, B and deionized water for washing was also put in the fluidics station.

In order to wash and stain the array, fluidics station 450 for AGCC was used by selecting protocol GenomeWide SNP 6_450. The protocol was then started and the instructions in the LCD were followed on the fluidics station. An array was inserted into the correct position of the fluidics station while the cartridge lever was in the down or eject position. After inserting, the cartridge lever was returned to the up or engaged position. After completion of the above stage, the fluidics protocol begins. The Fluidics Station dialog box at the workstation terminal and the LCD window displays the status of the washing and staining steps. After completion of the staining process, the microcentrifuge vials containing stain were removed and replaced with three empty vials. The arrays were removed from the fluidics station by pressing down the cartridge lever to the eject position. The presence of bubbles was checked by looking at the array window. If there were bubbles, one-half of the solution was manually removed and the array was manually filled with Array Holding Buffer. Scanning was done if there were no bubbles and if the arrays cannot be scanned immediately, it was stored at 4°C in the dark until ready for scanning. Scanning was performed within 24 hours. At the end of washing and staining processs, the fluidics station was shut down.

Figure 2.3 Fluidics station 450 for staining and washing arrays

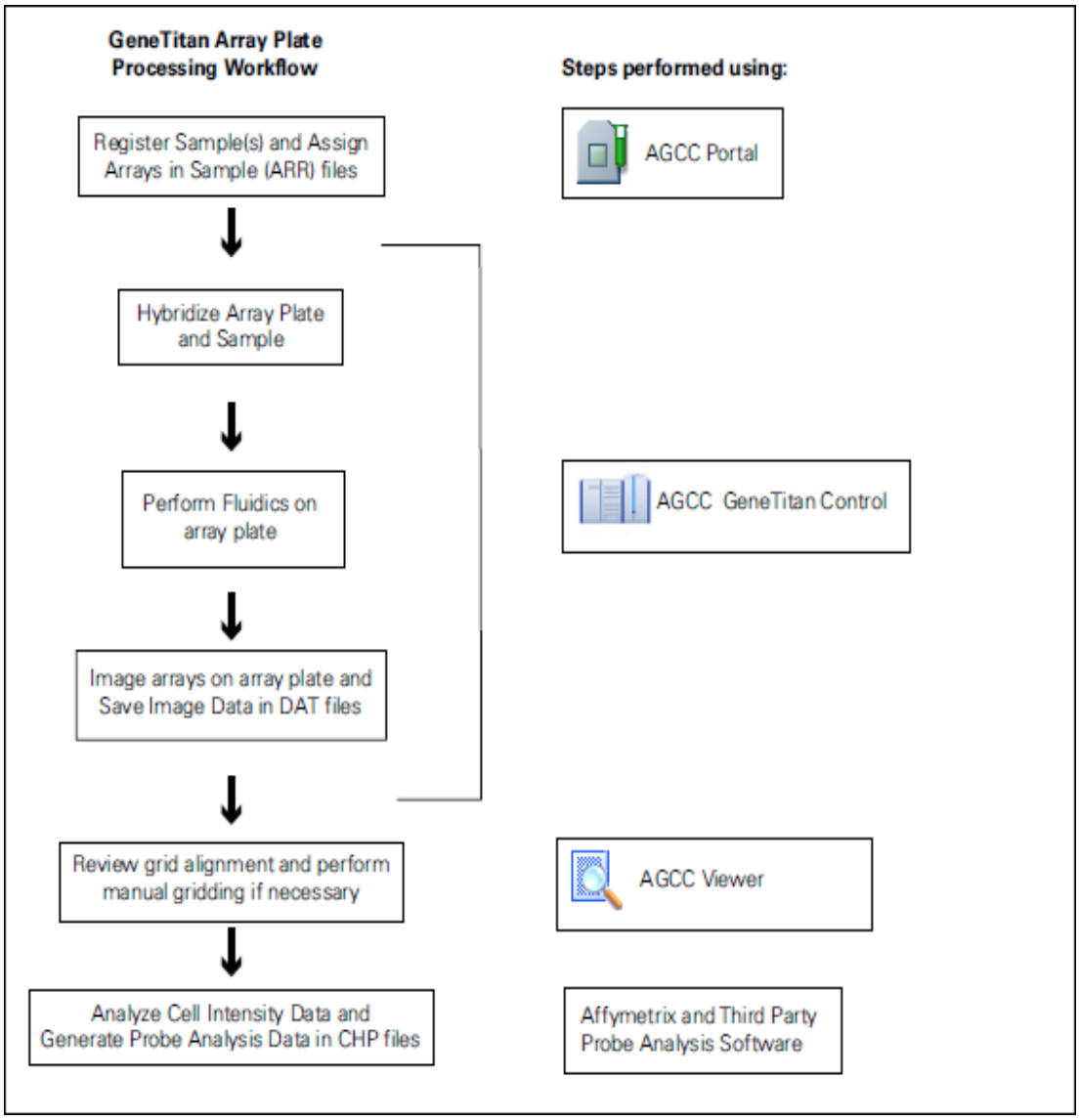

Figure 2.4 Array Plate Processing Workflow in GeneTitan System adapted from SNP 6.0 manual

2.4.11.10 Probe array scanning

The GeneChip Scanner 3000 7G was used in this experiment. It is controlled by the AGCC software. The scanner was turned on at least 10 min before use. The arrays were allowed to warm to room temperature before scanning if they were stored at 4°C. The glass surface of the array was cleaned with a tissue. Before scanning any excess fluid from around the septa on the back of the array cartridge was cleaned. Both septa were carefully covered with Tough Spots in order to prevent leaking of the fluid from the cartridge during scanning (Figure 2.5). The spots were pressed firmly to ensure they remain flat. An array was inserted into the scanner and tests the autofocus in order to ensure the spots do not interfere with the focus. If a focus error message was observed, the spot was removed and a new spot was applied. The arrays were scanned by selecting the sample name that corresponds to the array being scanned from AGCC software. The scan array image is saved in *.DAT files for further analysis once the scanning is complete and the scanner is switched off.

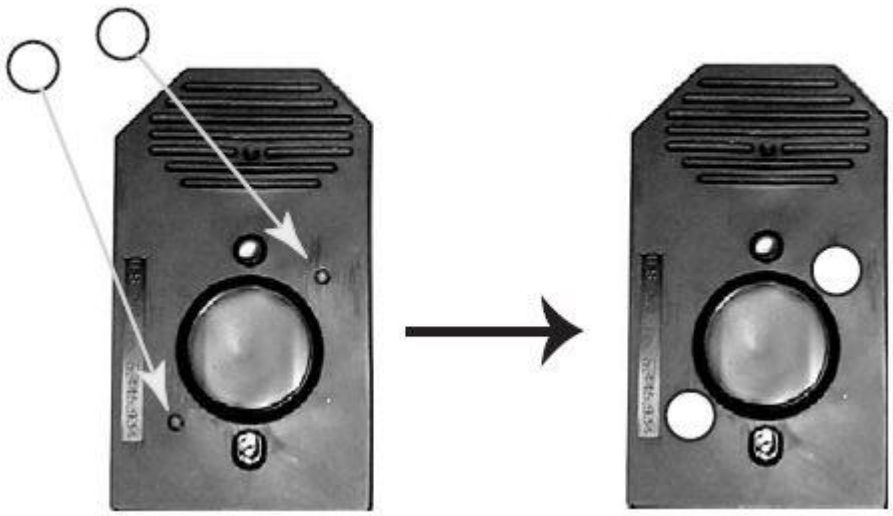

Figure 2.5 Applying Tough-Spots® to Arrays (SNP 6.0 manual)

2.4.11.11 Genotyping

The SNP Array 6.0 platform offers the genotype calling algorithm "Birdseed" to determine the genotypes of 906,600 SNPs (Affymetrix, Inc. http://www.affymetrix.com/index.affx). The Birdseed algorithm performs a multiple-chip analysis to estimate signal intensity for each allele of each SNP, fitting probe-specific effects to increase precision, and then makes genotype calls by fitting a Gaussian mixture model in the two-dimensional A-signal vs. B- signal space, using SNP-specific models to improve accuracy. In addition, this array also contains 945,826 copy number probes designed to interrogate CNVs in the genome. (Affymetrix, Inc. http://www.affymetrix.com/index.affx).

2.4.11.12 Data Quality Control (QC)

The filtering was done in order to get high quality genotype data and for enlarging the number of potentially associated SNPs. To achieve this poorly behaving SNPs were effectively eliminated with the SNP filtering parameters; those having a minor allele frequency (MAF) > 0.01, those deviating from Hardy-Weinberg equilibrium (HWE) p-value > 0.001 and SNP call rate > 95%.

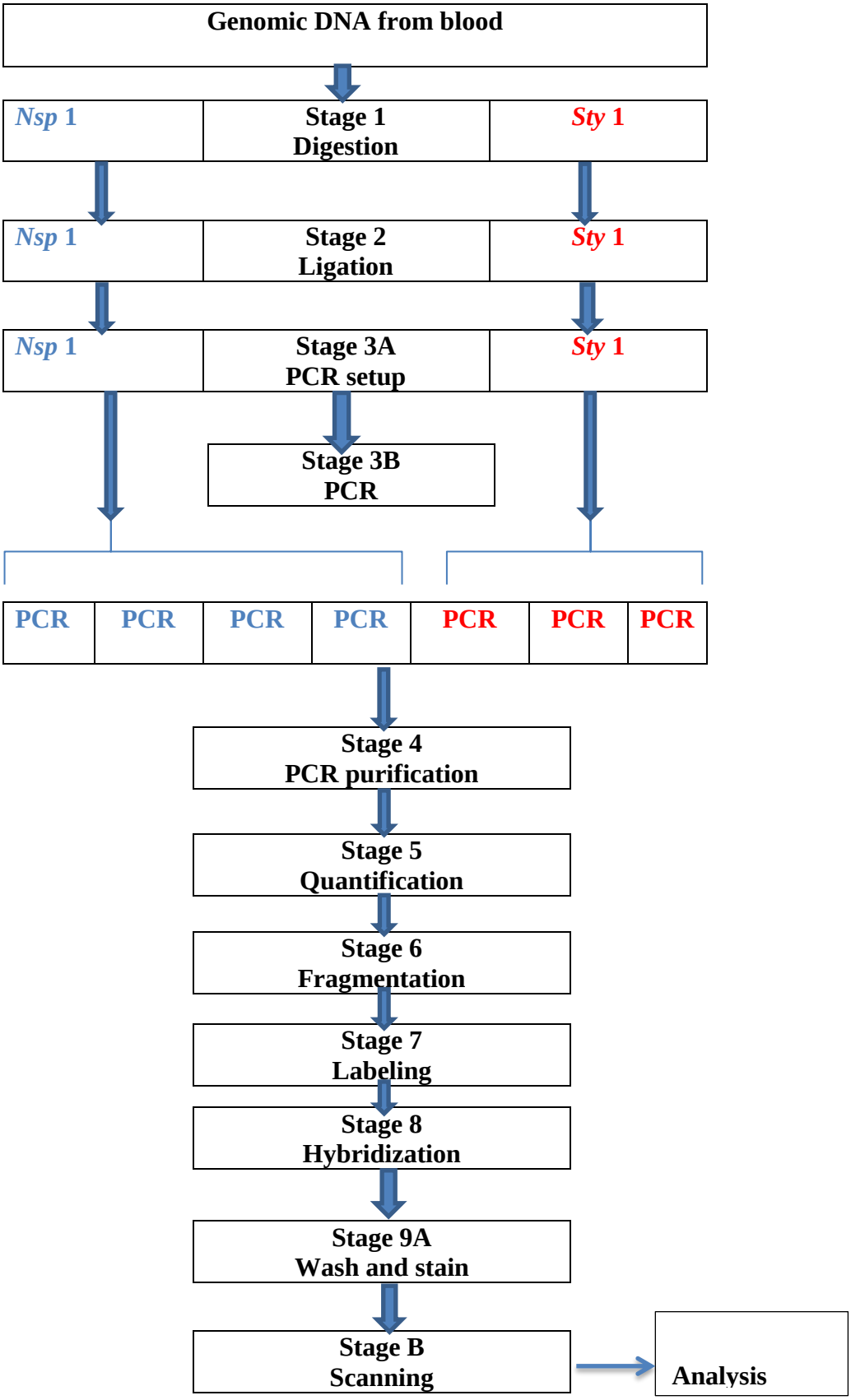

Figure 2.6 Cytogenetics copy number assay workflow overview

Chapter 3: Results

3.1 Phase I: *H. pylori cagA, dupA, babA*2 and *SabA* and *cagA* EPIYA motifs

3.1.1 Study population

A total of 226 patients (136 men and 90 women) with a mean age of 51.90 ± 13.58 years with a range of 20-86 years were recruited in the present study. These patient's consisted of three ethnic groups (Malay, Chinese, and Indian). The endoscopic findings revealed normal and abnormal findings which includes; normal, gastritis, GU and DU in 21 (9.3%), 186 (82.3%), 12 (5.3%) and 7 (3.1%) in patients respectively as shown in Figure 3.1.

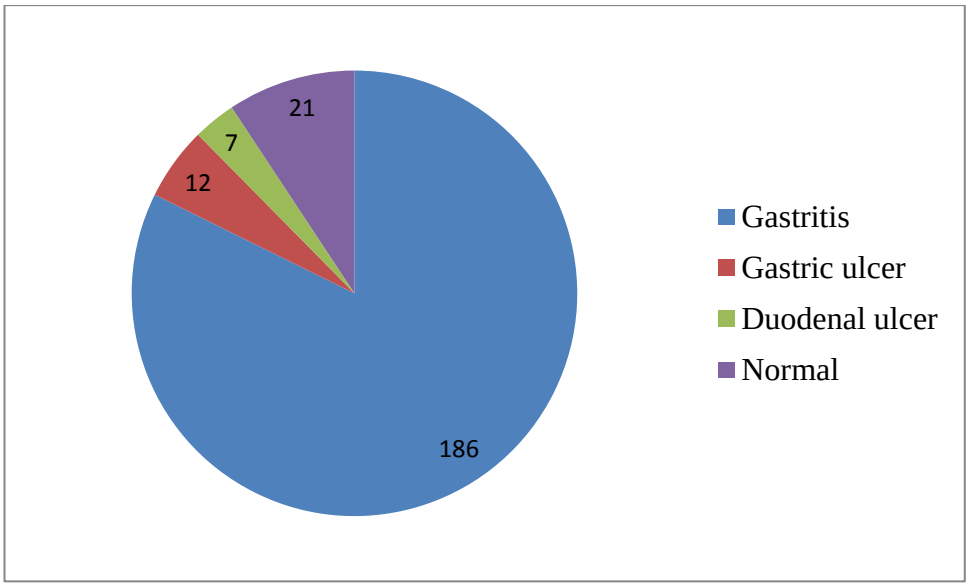

Figure 3.1 Distribution of the disease among patients

3.1.2 Detection of *H. pylori* infection by Rapid Urease Test (RUT)

The presence of *H. pylori* was mainly determined by RUT in this study. Out of the total 226 patients, 105 (46.5%) were confirmed positive for *H. pylori*. The mean age of infected patients was 54.48 ±12.94 years and age range of 26 to 86 years old. Fifty seven (54.3%) of the infected patients were males while fourty eight (45.7%) were females. Based on the endoscopic findings, 13 had normal OGDS finding, 78 patients had gastritis, nine had gastric ulcer and five had duodenal ulcer (Figure 3.2). The clinical presentation among the infected patients includes abdominal discomfort representing the highest 88, followed by heart burn 52, pains related meals 24 and vomiting 22.

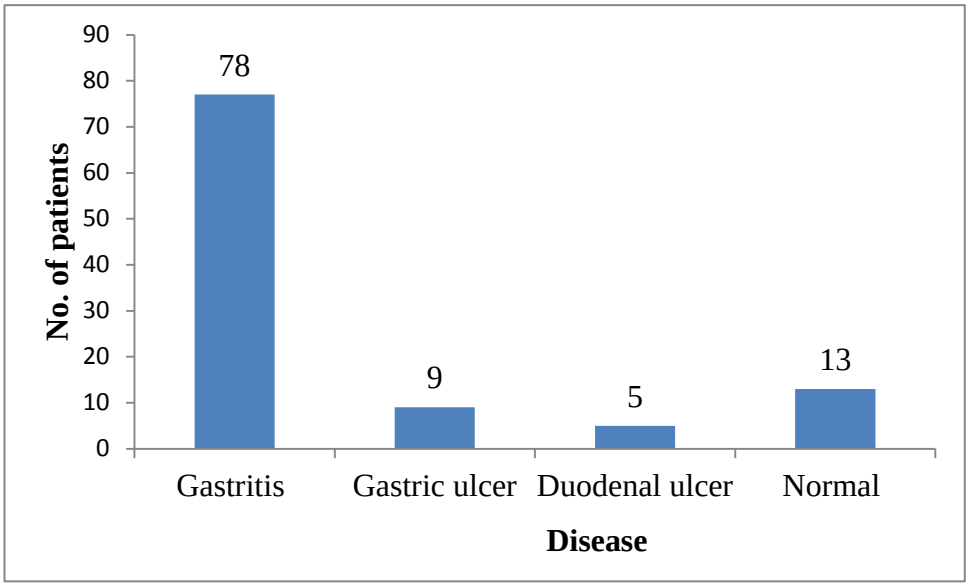

Figure 3.2 Distribution of the diseases among *H. pylori* positive patients

3.1.3 Detection of *H. pylori* infection by culture

Culture was performed on 81 samples out of which 33 samples tested positive and the rest were negative for *H. pylori*. The culture showed *H. pylori* as small translucent colonies as shown in the Figure 3.3

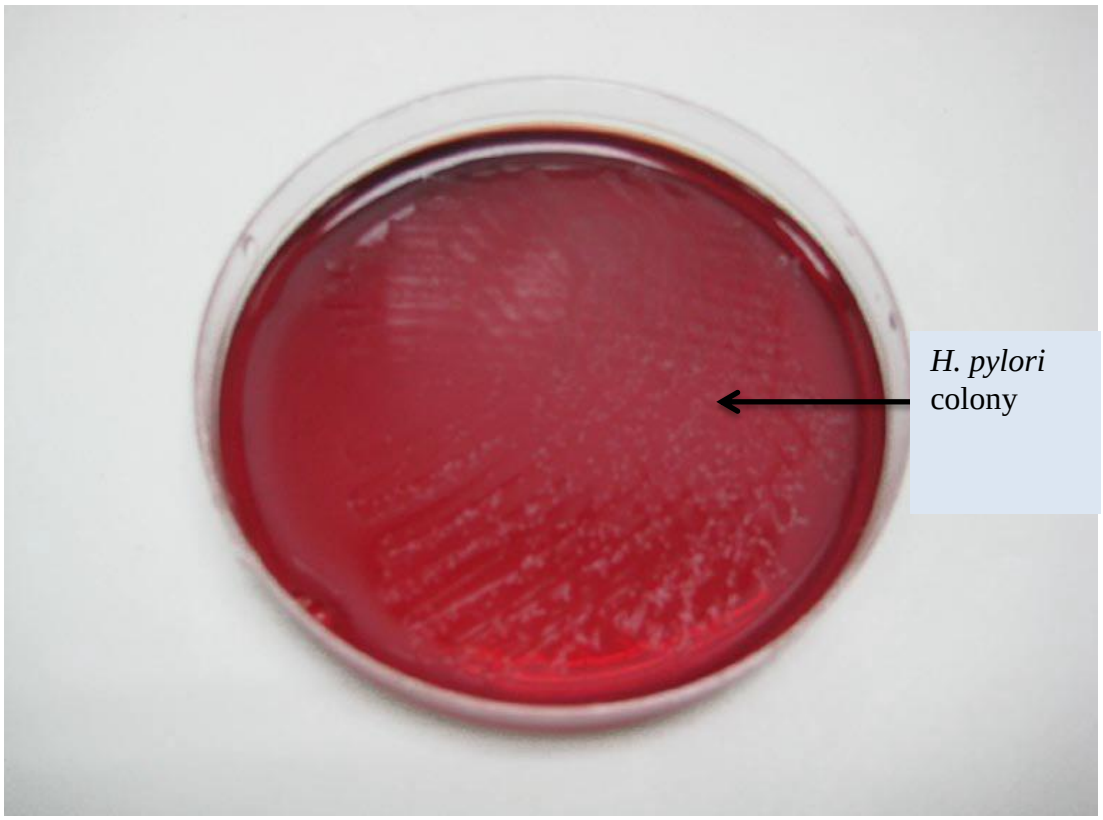

Figure 3.3 *H. pylori* growth on Columbia agar base supplemented with 7% laked horse blood and *H. pylori* Dent's selective supplements

3.1.4 Detection of *H. pylori* by Atlas *H. pylori* antigen test

A total of 59 patients, who consisted of 36 (61%) males and 23 (39%) females with a mean age of 51.2±13.3 years and ranging from 26-80 years were tested by *H. pylori* by Atlas *H. pylori* antigen test.

Out of the total, 24 patients were *H. pylori* positive and 35 were *H. pylori* negative by the gold standard method (rapid urease test). Atlas *H. pylori* antigen test was positive in 22 patients and negative in 35. Therefore, the sensitivity, specificity, PPV and NPV of Atlas *H. pylori* Antigen Test were 91.7%, 100%, 100% and 94.6% respectively. The diagnostic accuracy was 96.6% (Table 3.1).

Table 3.1 Sensitivity, specificity, positive and negative predictive values, and accuracy of Atlas *H. pylori* antigen test in the detection of *H. pylori* infection

	Atlas *H. pylori* antigen test n=59
True positive	22
True negative	35
False positive	0
False negative	2
Sensitivity (95% CI)	91.7% (72.9-98.7)
Specificity (95% CI)	100% (89.9-100)
Positive predictive value (PPV) (%)	100% (84.4-100)
Negative predictive value (NPV) (%)	94.6% (81.8-99.2)
Accuracy	96.6

95% CI = 95% confidence interval

Atlas *H. pylori* antigen test is considered negative when only one green band (control line) appears in the white central zone of the strip as indicated in Figure 3.4 while a positive test result is indicated by the appearance of green band at (control line) and red band in the zone marked T (result line) (Figure 3.5)

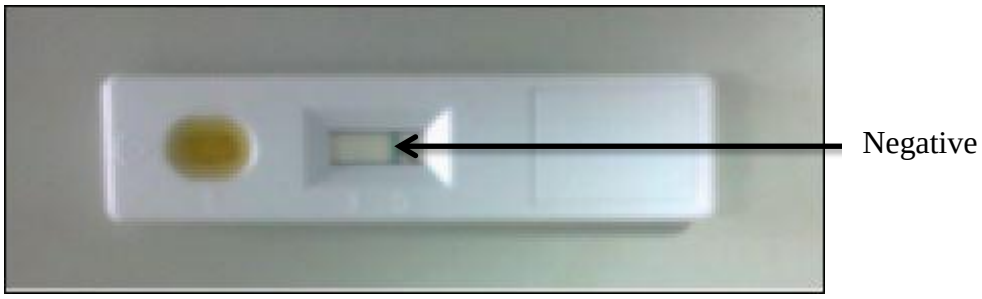

Figure 3.4 Negative Atlas *H. pylori* antigen test

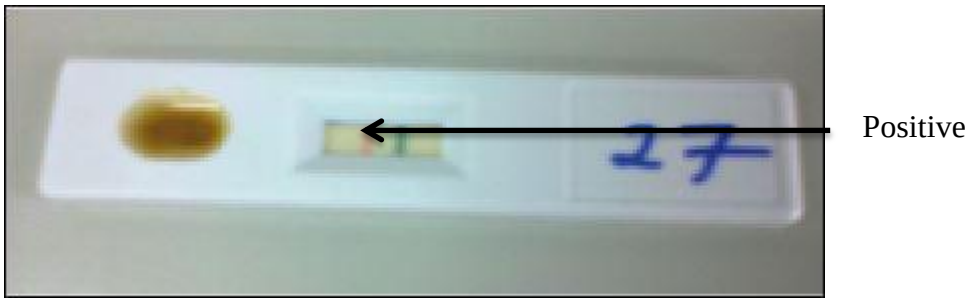

Figure 3.5 Positive Atlas *H. pylori* antigen test

3.1.5 Test agreement between culture, RUT and Atlas *H. pylori* stool antigen test

Table 3.2 and 3.3 shows the clinical agreement between culture, RUT and Atlas *H. pylori* stool antigen test for 59 samples. The kappa agreement between culture and RUT was ($k = 0.746$ and $P \leq 0.0001$ while the kappa agreement between culture and Atlas *H. pylori* stool antigen test was high ($k = 0.814$ and $P \leq 0.0001$).

Table 3.2 Clinical agreement between culture and RUT by kappa test (n=59)

Variable	**Culture n(%)**		**Total n(%)**	**Kappa (*k*)**	**p- value**
RUT	Negative	Positive			
Negative	34 (85.0)	1 (5.3)	35 (59.3)	0.746	<0.001
Positive	6 (15.0)	18 (94.7)	24 (40.7)		

Table 3.3 Clinical agreement between culture and Atlas *H. pylori* stool antigen test by kappa test (n=59)

Variable	**Culture n(%)**		**Total n(%)**	**Kappa (*k*)**	**p- value**
Atlas *H. pylori* antigen test	Negative	Positive			
Negative	36 (90.0)	1 (5.3)	37 (62.7)	0.814	<0.001
Positive	4 (10.0)	18 (94.7)	22 (37.3)		

3.1.6 Comparison of demographic characteristics of 105 patients and clinical outcomes based on social status and habits

In this study, the age was categorized into groups. Table 3.4 shows the demographic characteristics of 105 positive patients. Middle age group of 45-64 have highest prevalence *H. pylori* infection (55.2%). Gastritis is the most common clinical outcome among the three age groups, being highest in age group 45-64 (53.8%). However, there was no statistical significance between age groups. Male gender have high *H. pylori* infection rate (54.3%) as compared with female gender. Gastritis is the most common endoscopic findings in Male (56.4%) followed by DU (80.0%) while in Female also gastritis represents highest (43.6%) followed by GU (55.6%). GU is the highest among female gender.

Among the ethnic groups Malay has the highest infection rate (40.0%) with Indians slightly next (35.2%) and Chinese the lowest (24.8%). Gastritis is the predominant clinical outcome in all the races with Malay being highest (43.6%) and Chinese lowest (20.5%). In educational level those with high school have highest infection rate of (52.7%) while those with postgraduate level had the lowest (1.1%). Gastritis is the most common among high school level (52.9%).

The distribution of *H. pylori* infection varies among different occupation and its high in unemployed group (37.8%), slightly followed by manual workers (35.5%). Those who are in professional field have the lowest burden (8.9%). There is high rate of infection in non-smokers (75.0%) as compared with smokers (25.0%).

Table 3.4 Demographic characteristics of 105 patients with clinical outcomes (normal and abnormal OGDS findings) based on social status and habits

	Gastritis (78) n(%)	**GU (9) n (%)**	**DU (5) n(%)**	**Normal (13) n(%)**	**Total n(%)**
Age					
25-44	21 (26.9)	0 (0.0)	1 (20.0)	1 (7.7)	23 (21.9)
45-64	42 (53.8)	7 (77.8)	2 (40.0)	7 (53.8)	58 (55.2)
>65	15 (19.3)	2 (22.2)	2 (40.0)	5 (38.5)	24 (22.9)
Sex					
Male	44 (56.4)	4 (44.4)	4 (80.0)	5 (38.5)	57 (54.3)
Female	34 (43.6)	5 (55.6)	1 (20.0)	8 (61.5)	48 (45.7)
Race					
Malay	34 (43.6)	4 (44.4)	0 (0.0)	4 (30.8)	42 (40.0)
Indian	28 (35.9)	2 (22.2)	2 (40.0)	5 (38.5)	37 (35.2)
Chinese	16 (20.5)	3 (33.4)	3 (60.0)	4 (30.7)	26 (24.8)
Education					
None	17 (24.3)	2 (28.6)	3 (60.0)	0 (0.0)	22 (23.7)
High school	37 (52.9)	3 (42.8)	2 (40.0)	7 (63.6)	49 (52.7)
College	12 (17.1)	2 (28.6)	0 (0.0)	2 (18.2)	16 (17.2)
University	3 (4.3)	0 (0.0)	0 (0.0)	2 (18.2)	5 (5.3)
Postgraduate	1 (1.4)	0 (0.0)	0 (0.0)	0 (0.0)	1 (1.1)
Occupation					
Unemployed	23 (35.4)	5 (55.6)	1 (25.0)	5 (41.7)	34 (37.8)
Manual	23 (35.4)	2 (22.2)	2 (50.0)	5 (41.7)	32 (35.5)
Administrative	13 (20.0)	2 (22.2)	0 (0.0)	1 (8.3)	16 (17.8)
Professional	6 (9.2)	0 (0.0)	1 (25.0)	1 (8.3)	8 (8.9)
Smoking status					
Smoker	18 (27.7)	0 (0.0)	2 (50.0)	2 (15.4)	22 (25.0)
Non smoker	47 (72.3)	6 (100)	2 (50.0)	11 (84.6)	66 (75.0)

GU: Gastric ulcer, DU- duodenal ulcer

3.1.7 Distribution of *cagA*, *babA2*, *dupA* and *sabA* according to ethnicity

The 297 bp PCR product which is apt at indicating the presence of the *cagA* gene as shown in Figure 3.7 was detected in 73 (69.5%) of the biopsy samples. The detection of *cagA* gene among the three ethnic groups; namely Malay, Indian and Chinese were 26 (35.6%), 29 (39.7%) and 18 (24.7%) respectively (Figure 3.6). The Indian population potrayed the highest distribution of *cagA* gene compared with others.

The distribution of *sabA* gene among Malay, Indian and Chinese was 20 (43.5%), 18 (39.1%) and 8 (17.4%) respectively as indicated in Figure 3.6. *sabA* gene showed 264 bp after running the gel (Figure 3.7).

The *babA2* gene amplified 271 bp (Figure 3.8) was discovered in 43 (41.0%). The distribution of the *babA2* gene among Malay, Indian and Chinese was 17 (39.5%), 14 (32.6%) and 12 (27.9%) respectively (Figure 3.6). *dupA* gene which was 197 bp as indicated in Figure 3.9 was also found in overall 24 (22.9%) of the biopsy samples. The distribution of *dupA* gene among Malay, Indian and Chinese inhabitants was 9 (37.5%), 9 (37.5%) and 6 (25.0%) respectively (Figure 3.6).

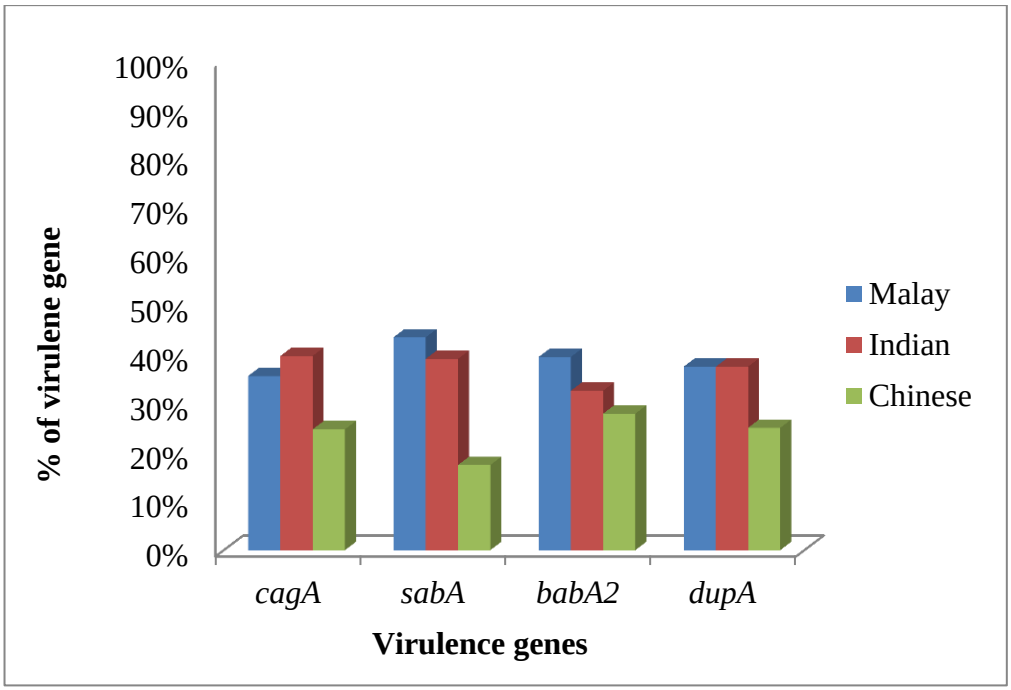

Figure 3.6 Distribution of *cagA, babA2, dupA* and *sabA* gene by ethnicity

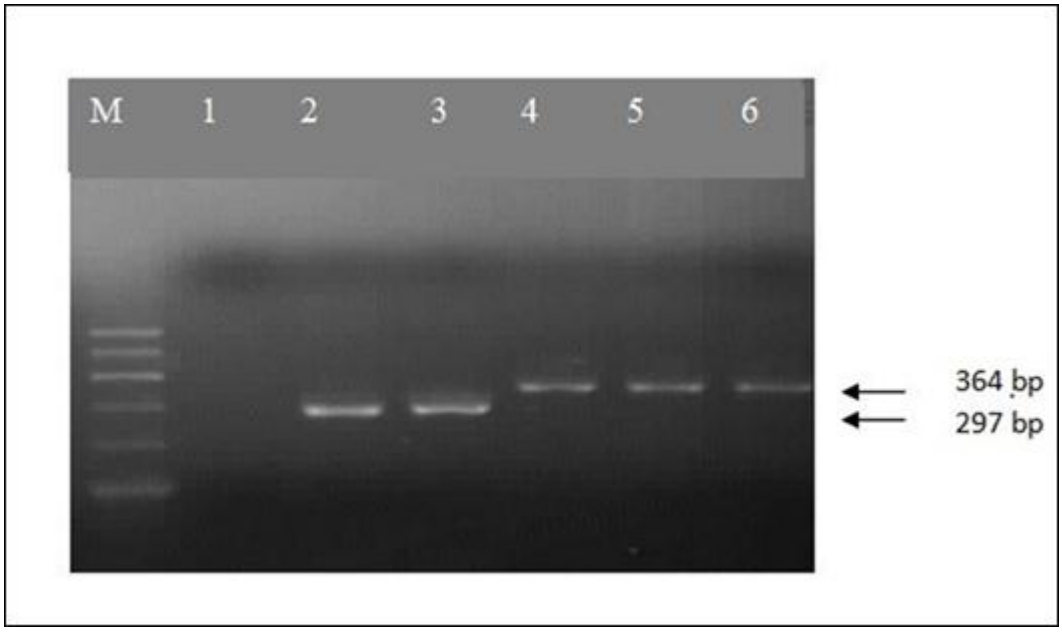

Figure 3.7 PCR product of of *cagA* and *sabA* genes

Lane M, 100 bp DNA marker; Lane 1, blank (negative control without DNA); Lanes 2 and 3 are *cagA* positive *H. pylori* strains (297bp); Lanes 4, 5 and 6 are *SabA* positive *H. pylori* strains (364bp).

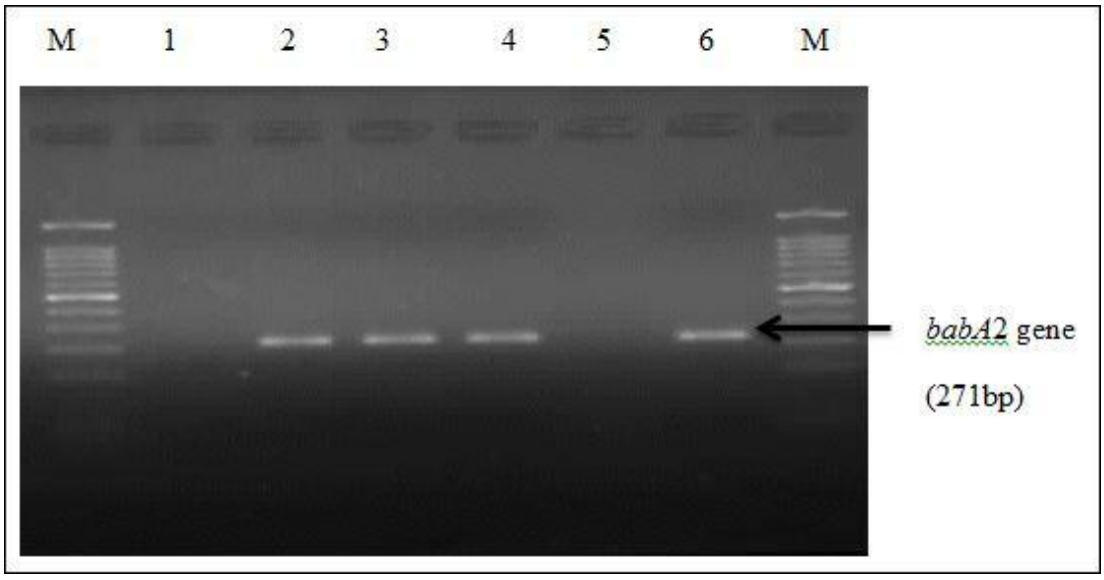

Figure 3.8 PCR product of *babA2* gene

Lane M, 100-bp DNA marker; lanes 1, blank (negative control without DNA); lane 5, negative sample (negative *H. pylori* clinical sample); Lane 2, *H. pylori* positive control (strain ATCC 26695) and Lanes, 3, 4 and 6, *H. pylori* as positive strain with *babA2* gene (271 bp).

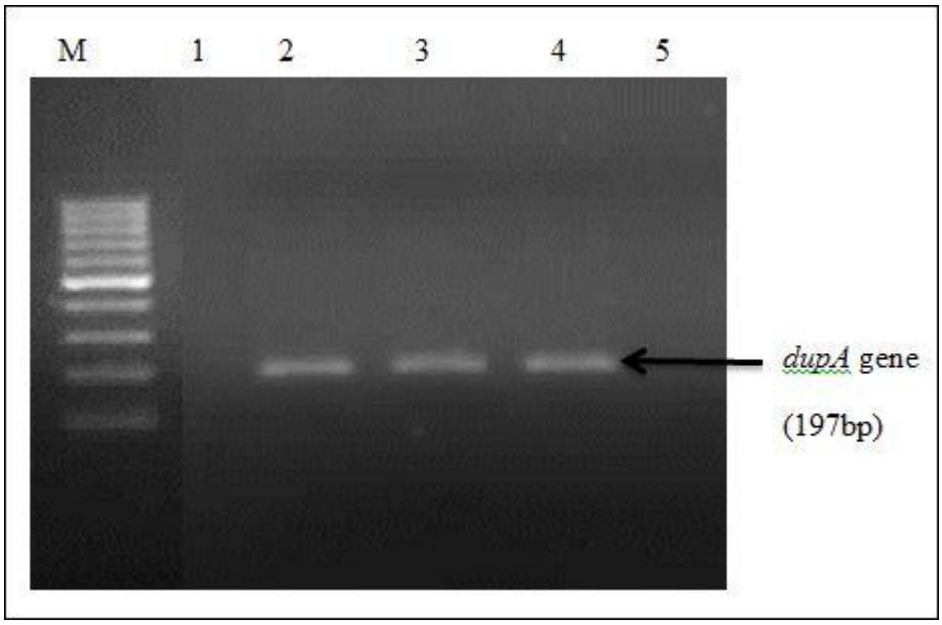

Figure 3.9 PCR product of *dupA* gene

Lane M, 100-bp DNA marker; lanes 1, blank (negative control without DNA); lane 5, negative sample (negative *H. pylori* clinical sample); and Lanes, 2, 3 and 4, *H. pylori* as positive strain with *dupA* gene (197 bp).

3.1.8 Distribution of *H. pylori cagA, babA2, dupA* and *sabA* genes individually with its clinical outcome

The distribution of *cagA babA2, dupA* and *sabA* among different gastroduodenal diseases were shown in Table 3.5. Out of the 105 *H. pylori* positive patients, 69. 5% were positive for *cagA* gene while 30.5% were negative. *cagA* gene is higher in gastritis patients (76.7%) as compared with GU (11.0%), DU (4.1%) and normal groups (8.2%). Similarly, the *babA2* gene was present in 41% of the positive cases and absent in 59% and it is most prevalent in gastritis (74.4%) patients as compared to the other diseases. *dupA* gene was positive in 22.9% of the total while 77.1% were negative. The *dupA* gene was most frequent in the gastritis (75.0%) patients as compared to others. *sabA* gene is also most common in gastritis patients (78.3%) although low in GU and DU patients. 43.8% of the *H. pylori* infected patients had *sabA* gene while 56.2% are negative for *sabA* gene. Despite this distribution, there was no significant difference between *cagA, babA2 dupA* and *sabA* genes and clinical outcome.

3.1.9 Study of combined virulence genes and clinical outcome

A combination of *cagA, babA2 dupA* and *sabA* was detected in 8 biopsy samples and a combination of *cagA* and *babA2* was found in 38 biopsy samples. 21 patients had a combination of *cagA* and *dupA*. A total of 39 biopsy samples were positive for *cagA* and *sabA* as shown in (Table 3.6). There was no significant difference noted between the combinations and clinical outcome

Table 3.5 Distribution of *cagA, babA2, dupA* and *sabA* and clinical outcome in *H. pylori* infected patients.

Virulence genes	**Gastritis (78) n(%)**	**GU (9) n (%)**	**DU (5) n (%)**	**Normal (13) n (%)**	**Total 105 (%)**	***P* value**
cagA+	56 (76.7)	8 (11.0)	3 (4.1)	6(8.2)	73 (69.5)	0.146
cagA -	22 (68.8)	1 (3.1)	2 (6.2)	7(21.9)	32 (30.5)	
babA2+	32 (74.4)	5 (11.6)	3 (7.0)	3 (7.0)	43 (41.0)	0.290
babA2-	46 (74.2)	4 (6.5)	2 (3.2)	10 (16.1)	62 (59.0)	
dupA+	18 (75.0)	1 (4.2)	2 (8.3)	3 (12.5)	24 (22.9)	0.700
dupA-	60 (74.1)	8 (9.9)	3 (3.7)	10 (12.3)	81 (77.1)	
sabA+	36 (78.3)	4 (8.7)	3 (6.5)	3 (6.5)	46 (43.8)	0.400
sabA-	42 (71.2)	5 (8.5)	2 (3.4)	10 (16.9)	59 (56.2)	

*Fisher's exact applied

Table 3.6 Combined *cagA, babA2 dupA* and *sabA* genotypes and clinical outcome

Virulence genes	**Gastritis n (%)**	**GU n (%)**	**DU n (%)**	**Normal n (%)**	***P* value**
cagA+/*babA2*+/ *dupA*+/ *sabA*+	6 (13.0)	0 (0.0)	1 (33.3)	1 (25.0)	0.328
cagA-/*babA2*-/ *dupA*-/ *sabA*-	40 (87.0)	6 (100.0)	2 (66.7)	3 (75.0)	
cagA+/*babA2*+	28 (60.9)	5 (83.3)	2 (66.7)	3 (75.0)	0.842
cagA-/*babA2*-	18 (39.1)	1 (16.7)	1 (33.3)	1 (25.0)	
cagA+/*dupA*+	16 (34.8)	1 (16.7)	2 (66.7)	2 (50.0)	0.485
cagA-/*dupA*-	30 (65.2)	5 (83.3)	1 (33.3)	2 (50.0)	
cagA+/*sabA*+	29 (63.0)	4 (66.7)	3 (100.0)	3 (75.0)	0.759
cagA-/*sabA*-	17 (37.0)	2 (33.3)	0 (0.0)	1 (25.0)	

*Fisher's exact applied

3.1.10 Detection of *cagA* EPIYA motifs

Out of 105 specimens confirmed to have *H. pylori* infection, 73 (69.5%) were *cagA* positive consisting of 39 (53.42%) males and 34 (46.58%) females. The mean age was 53.37 ±12.18 years. Based on the endoscopic findings, 6 had normal finding, 56 patients had gastritis, 8 had GU and. 3 DU. Males have higher prevalence of gastritis (55.36%) and DU (100%) in comparison with females as indicated in Table 3.7.

3.1.10.1 *CagA* genotypes among patients of different age and ethnic groups

The prevalence of the Western type (54. 79%) was higher than the East Asian type (38.36%) and (6.85%) were unclassified type (A-B). The EPIYA *cagA* genotypes is higher in patients with age group between 44-64 years, accounting for (60.27%) and lower (17.81%) in elderly above >65 old (Table 3.7).

The distribution of *cagA* EPIYA motif varies among patients from different ethnic groups and with variant diseases. Majority of Chinese patients were infected with *H. pylori* strains carrying *cagA* type A-B-D 16 (88.9%), whereas infection with *H. pylori* strains carrying *cagA* type A-B-C were largely detected in Indians 24 (82.8%) and Malays 14 (53.8%). There were statistically significant difference ($P<0.001$) between ethnicity and *cagA* EPIYA motifs (Table 3.7).

Table 3.7 Diversity of *cagA* genotypes among patients of different age and ethnic groups

***cagA* genotype**	**East Asian type (ABD)**	**Western type (ABC)**	**Unclassified type**	**n (%)**	****P* value**
Age					
25-44	3 (10.71)	12 (30.0)	1 (20.0)	16 (21.92)	
45-64	18 (64.29)	23 (57.50)	3 (60.0)	44 (60.27)	0.283
>65	7 (25.0)	5 (12.50)	1(20.0)	13 (17.81)	
Ethnic group					
Indian	4 (13.8)	24 (82.8)	1 (3.4)	29 (39.73)	
Malay	8 (30.8)	14 (53.8)	4 (15.4)	26 (35.62)	0.001
Chinese	16 (88.9)	2 (11.1)	0 (0.0)	18 (24.66)	

*Fisher's exact applied

3.1.10.2 Distribution of *cagA* EPIYA motif and clinical outcome

The examples of PCR amplification of EPIYA motifs are shown in Figure 3.10. The distribution of *cagA* EPIYA motifs among patients with different clinical outcomes is shown in Table 3.8. Out of 73 strains, 5 (6.85%) had the unclassified type, 40 (54. 79%) had ABC and 28 (38.36%) had the ABD type.

Multiple EPIYA motifs were found only in Western type. The Western EPIYA motif detected were ABCC 11 (15.06%) and ABCCC 2 (2.74%). Those with ABD type had highest distribution in gastritis patients 20 (35.71%). DU and GU case isolated had low distribution in both ABC and ABD type.

Table 3.8 Distribution of gender and *cagA* EPIYA motifs and clinical outcomes in *H. pylori cagA* positive infected patients.

	Clinical outcome					
Gender	**Gastritis n (%)**	**GU n (%)**	**DU n (%)**	**Normal n (%)**	**Total n (%)**	***P* value**
Male	31 (55.36)	4 (50)	3 (100)	1 (16.67)	39 (53.42)	0.106
Female	25 (44.64)	4 (50)	0 (0)	5 (83.33)	34 (46.58)	
***cagA* EPIYA type**						
Western type						
A-B-C	20 (35.71)	3 (37.5)	2 (66.67)	2 (33.33)	27 (36.99)	0.729
A-B-C-C	9 (16.07)	2 (25)	0 (0)	0 (0)	11 (15.06)	
A-B-C-C-C	2 (3.57)	0 (0)	0 (0)	0 (0)	2 (2.74)	
East Asian type						
A-B-D	20 (35.71)	3 (37.5)	1 (33.33)	4 (66.67)	28 (38.36)	0.542
Unclassified type						
A-B	5 (8.93)	0 (0)	0 (0)	0 (0)	5 (6.85)	

GU = gastric ulcer; DU = duodenal ulcer

*Pearson Chi-square test applied

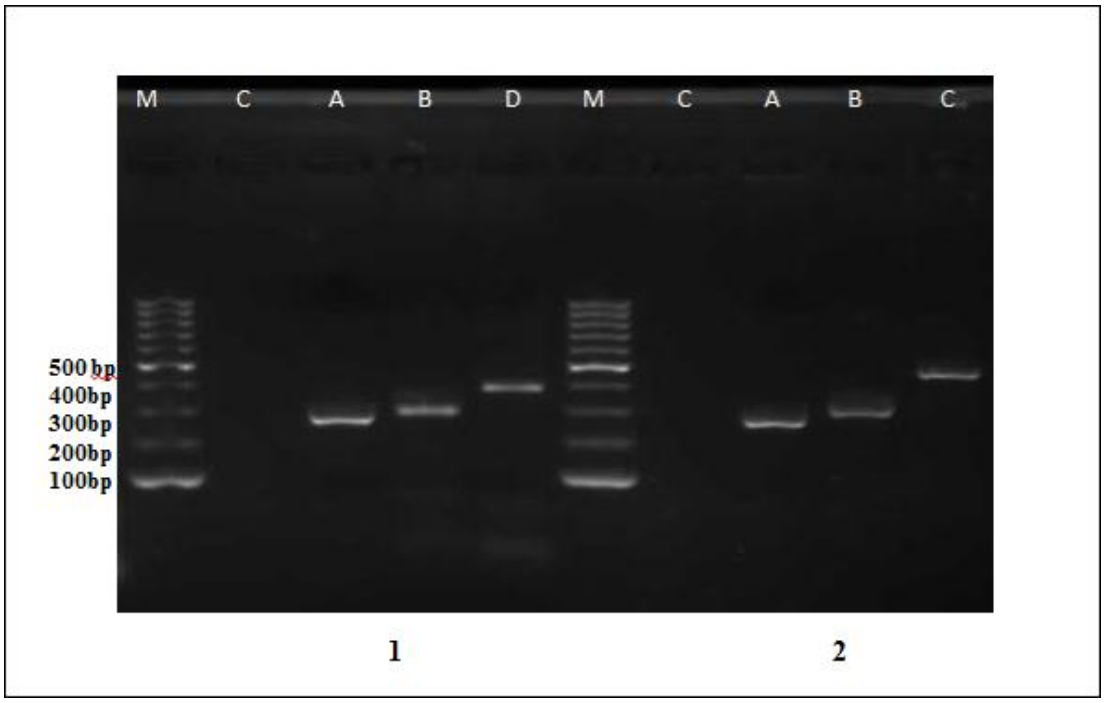

Figure 3.10 PCR detection of *cagA* EPIYA motif from *H. pylori* strains.

1: Lane M, 100‑bp DNA marker; Lane C, blank (negative control without DNA) ; Lane A, B and D, ABD strain (495 bp)

2: Lane M, 100‑bp DNA marker; Lane C, blank (negative control without DNA); Lane A, B and C, ABC strain (501 bp).

3.1.10.3 Sequencing of the 3' Variable Region of *cagA* Gene

Nucleotide sequencing of the purified products of the 3' variable region of *cagA* gene was performed for 11 randomly selected strains including 7 from Gastritis, 2 from GU and 2 from DU patients (Table 3.9).

3.1.10.3.1 Identification of Amino Acids of 3' Variable Region of *cagA*

Sequence analyses for 5 samples confirmed that three types of EPIYA motifs for ABC type and one sample had ABCC:

-EPIYA-A for EPIYAKVNKKK;

-EPIYA-B for EPIYAQVAKKVNAKI; and

-EPIYA-C for EPIYATIDDLGG

Five strains posses the East Asian type of EPIYA-D

-EPIYA-A for EPIYAQVNKKK;

-EPIYA-B for EPIYAQVAKKVSA; and

- EPIYA –D for EPIYATIDFDEANQAG

The result confirmed that PCR methods correctly classified the EPIYA motif types for both ABC amd ABD. Sequence alignment results showed that EPIYA-B (strains 89) carried *cagA* with a modified EPIYA motif (EPIYT) which had a threonine residue instead of alanine residue. In contrast, all strains sequences in EPIYA-C contained exactly EPIYA sequences. The alignment of partial *cagA* peptide sequences showing the EPIYA motifs of *H. pylori* isolates from patients with DU, GU and gastritis with the reference strain in GenBank (Acc. No. AEF97751 and AB246742) are shown in Figure 3.11 for EPIYA ABC and Figure 3.12 for EPIYA ABD.

Table 3.9 The selected strains for nucleotide sequence of the *cagA* variable region

Strain	**Sex**	**Age (year)**	**Ethnicity**	**EPIYA motif**	**Disease**
Malaysia-72	Male	70	Indian	ABC	DU
Malaysia-83	Female	41	Indian	ABC	Gastritis
Malaysia-84	Male	47	Malay	ABC	Gastritis
Malaysia-94	Female	54	Indian	ABC	Gastritis
Malaysia-113	Male	36	Indian	ABC	DU
Malaysia-89	Male	50	Malay	ABCC	GU
Malaysia-92	Female	26	Malay	ABD	Gastritis
Malaysia-100	Male	57	Malay	ABD	Gastritis
Malaysia-107	Male	61	Chinese	ABD	Gastritis
Malaysia-111	Male	57	Chinese	ABD	Gastritis
Malaysia-112	Male	70	Chinese	ABD	GU

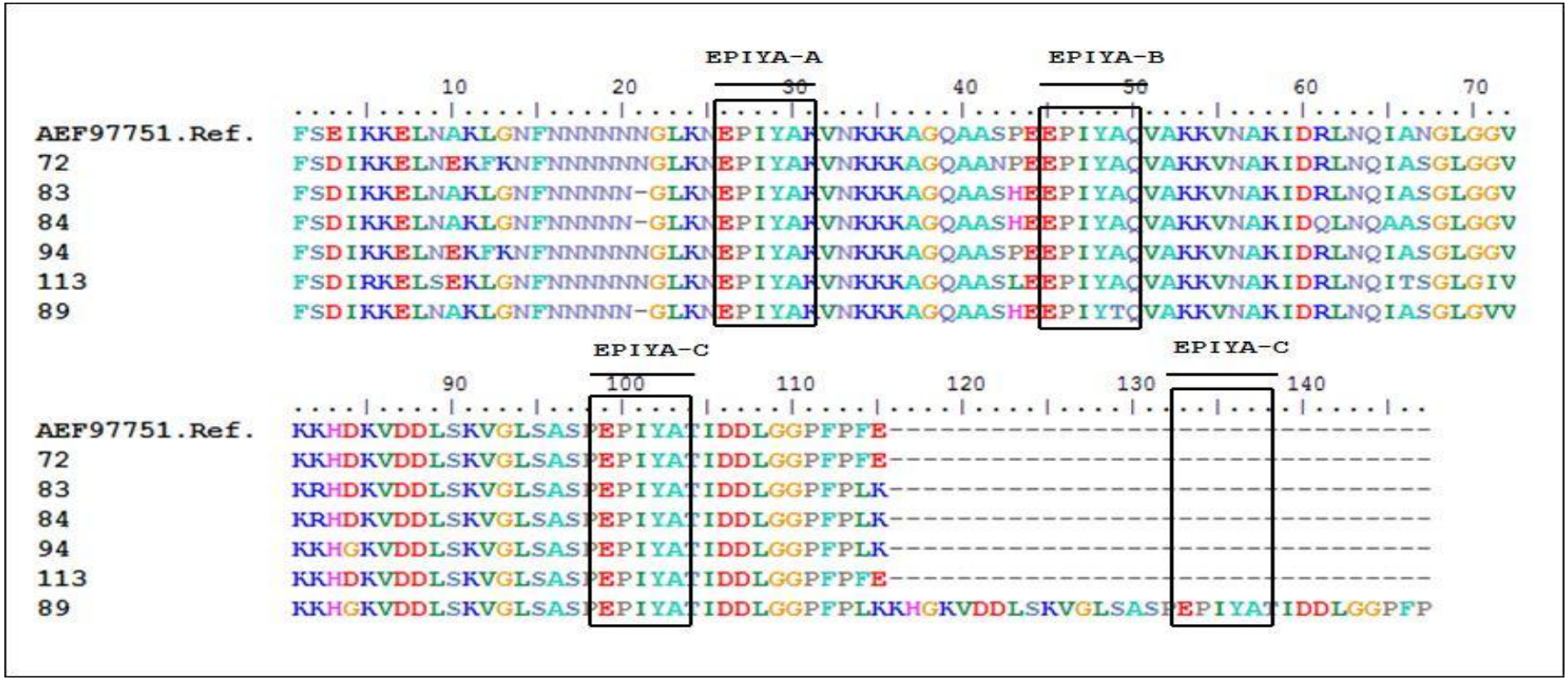

Figure 3.11 Alignment of partial *cagA* peptide sequences showing reference strain AEF97751 and EPIYA ABC type (sample 72, 83, 84, 94 and 113) and ABCC type (sample 89)-EPIYA-A for EPIYAKVNKKK; EPIYA-B for EPIYAQVAKKVNAKI; and EPIYA-C for EPIYATIDDLGG.

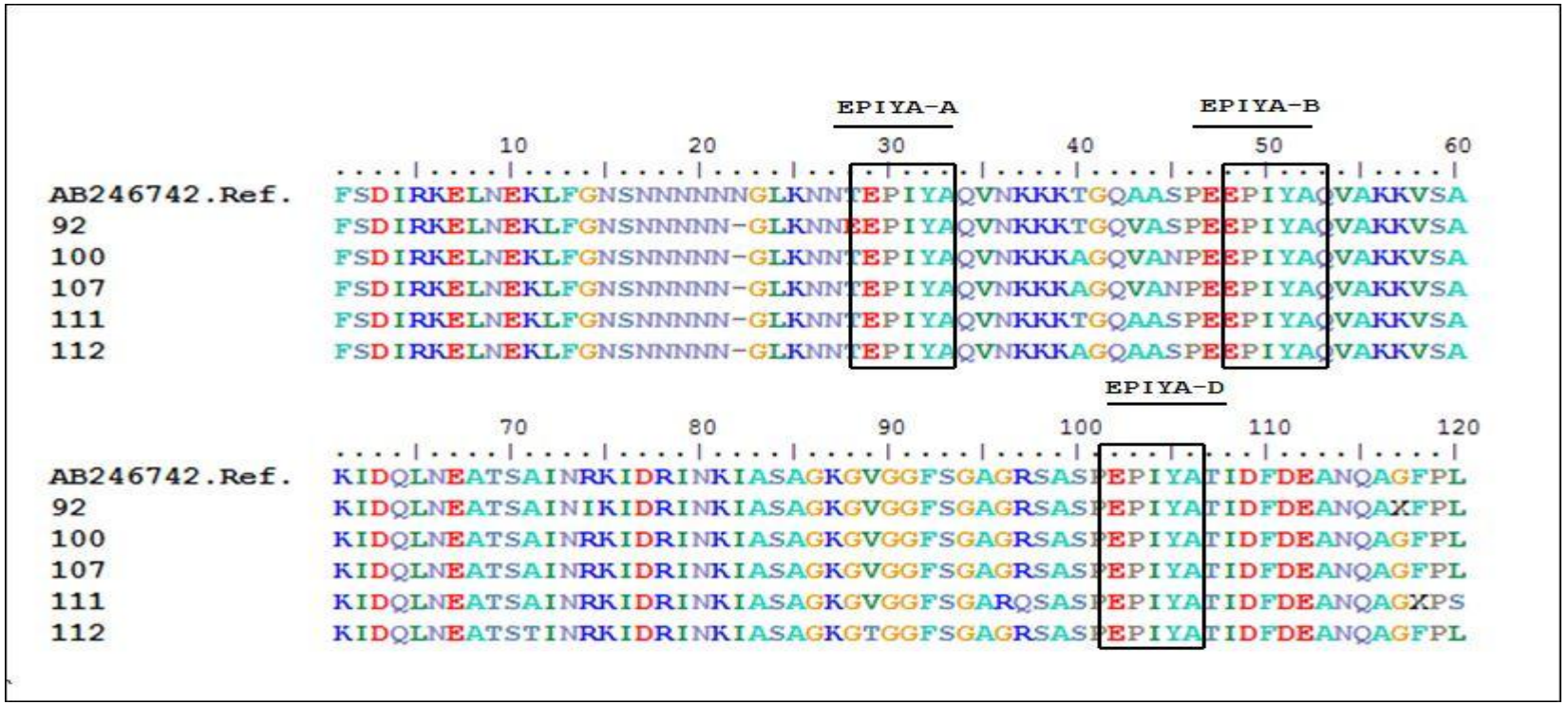

Figure 3.12 Alignment of partial *CagA* peptide sequences showing reference strain AB246742 and EPIYA ABD type (sample 92, 100, 107, 111 and 112)- EPIYA-A for EPIYAQVNKKK; EPIYA-B for EPIYAQVAKKVSA and EPIYA –D for EPIYATIDFDEANQAG.

3.2 Phase II: Genome wide association study (GWAS)

3.2.1 Study population

A total of 80 (42 *H. pylori* positive and 38 *H. pylori* negative) third generation patients with a mean age of 49.87 ± 12.335 years (age range 20-75 years) were included into this study after fulfilling inclusion criteria (Table 3.10). Male *H. pylori* positive patients represents 23 which is slightly higher than the female counterpart which is 19 patients. Indian ethnic group had highest positive case (22) followed by Malay (11) and Chinese (9).

Prior to Microarray protocol, the DNA of the patients was checked by Nanodrop and electrophoresis as shown in Figure 3.13. A high molecular weight DNA indicates the good quality of the extracted DNA. Once the DNA quality was to the expected quality, then microarray protocol was conducted. According to Genome Wide Human SNP 6 assay, PCR products should be in the range of 250 and 2000 bp, in the current study, PCR product in the required range was obtained (Figure 3.14). After purification, quantification of PCR product was done and only samples that have required concentration and purity were proceeded to fragmentation step.

Table 3.10 Distribution of both cases and control according to gender and ethnicity

	Cases (*H. pylori* positive)	**Control (*H. pylori* negative)**
Gender		
Male	23	28
Female	19	10
Total	**42**	**38**
Ethnicity		
Indian	22	19
Malay	11	10
Chinese	9	9
Total	**42**	**38**

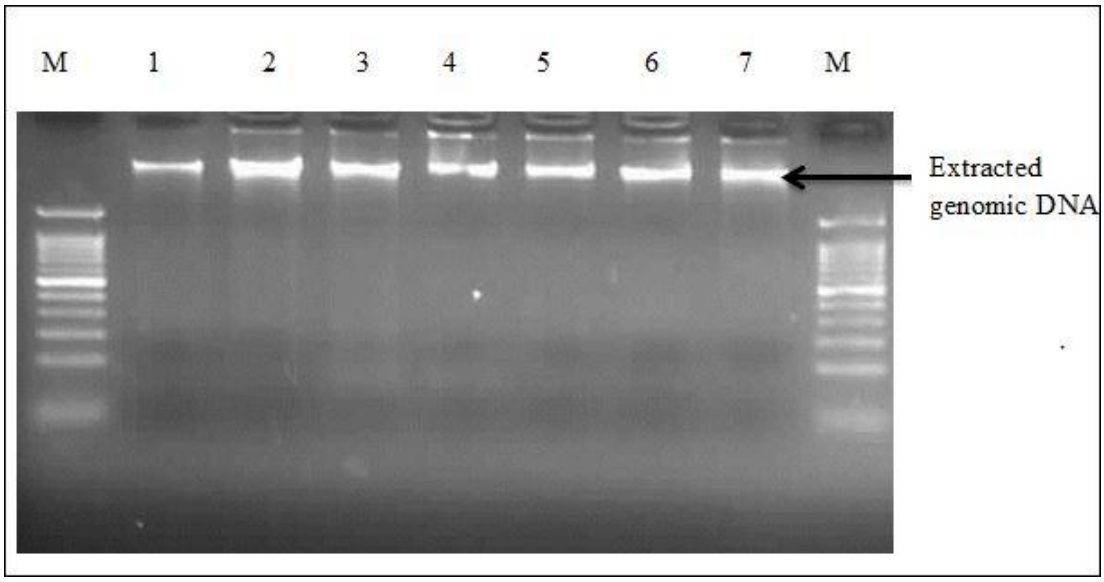

Figure 3.13 Gel electrophoresis picture showing extracted genomic DNA of some samples Lane M, 100bp ladder; Lane 1-8, DNA samples.

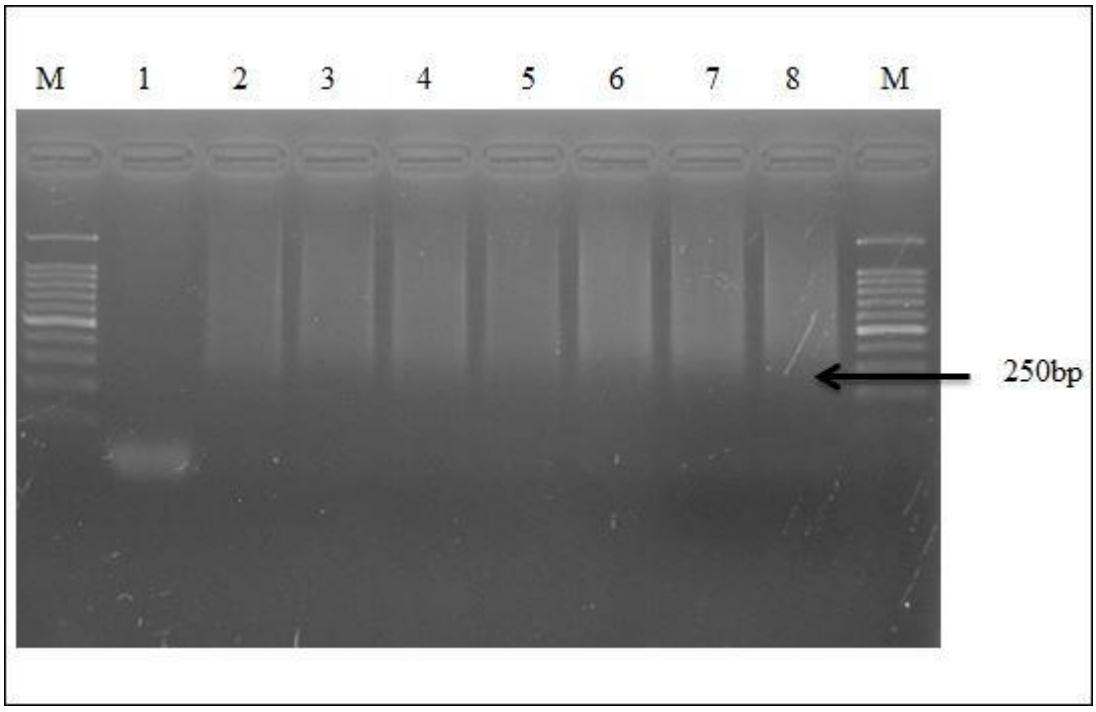

Figure 3.14 Gel electrophoresis of PCR product

Lane M, 100 bp ladder; Lane 1, negative control and lanes 2-8, PCR products 2% agarose gel at 120V for 1 hour with an average band size of between 250 and 2000 bp.

3.2.2 Determining QC call rate generated by genotype console software

Out of the total 80 samples, only 72 samples (38 cases and 34 controls) passed the required QC call rate of 0.4 and above (Table 3.11). According to Genome Wide Human SNP 6 assay protocol only samples that pass the QC rate were included in the analysis. The GTC software computes QC rate, those that pass 0.4 are indicated as in bounds while those below 0.4 are out bounds. Figure 3.15 shows example of samples that have call rate 90% and above.

Table 3.11 QC call rate generated by genotype console software

No	File	*Bounds	Contrast QC	QC Call Rate	Computed Gender	# CHP/CEL	File Date
1	*H. pylori*_case5_(GenomeWideSNP_6).CEL	In	0.87	95.57	Male	1	12/21/2014 10:11 AM
2	*H. pylori*_case 11_(GenomeWideSNP_6).CEL	In	1.28	94.84	female	1	12/21/2014 9:16 AM
3	*H. pylori*_case 13_(GenomeWideSNP_6).CEL	In	1.43	94.87	female	1	12/21/2014 9:50 AM
4	*H. pylori*_case 19_(GenomeWideSNP_6).CEL	In	1.06	95.40	female	1	12/21/2014 10:03 AM
5	*H. pylori*_case 21_(GenomeWideSNP_6).CEL	In	1.16	95.67	Male	1	12/21/2014 10:05 AM
6	*H. pylori*_case 38_(GenomeWideSNP_6).CEL	In	1.63	95.10	Male	1	12/21/2014 10:06 AM
7	*H. pylori*_case 43_(GenomeWideSNP_6).CEL	In	1.12	93.05	Male	1	12/21/2014 10:08 AM
8	*H. pylori*_case 45_(GenomeWideSNP_6).CEL	In	0.72	91.66	Male	1	12/21/2014 10:08 AM
9	*H. pylori*_case 46_(GenomeWideSNP_6).CEL	In	0.77	92.85	female	1	12/21/2014 10:10 AM
10	*H. pylori*_case 52_(GenomeWideSNP_6).CEL	In	0.45	89.91	female	1	12/21/2014 10:12 AM
11	*H. pylori*_case 53_(GenomeWideSNP_6).CEL	In	1.84	95.50	female	1	12/21/2014 10:13 AM
12	*H. pylori*_case 58_(GenomeWideSNP_6).CEL	In	1.16	94.37	female	1	12/21/2014 10:14 AM
13	*H. pylori*_case 59_(GenomeWideSNP_6).CEL	In	1.11	95.23	female	1	12/21/2014 10:15 AM
14	*H. pylori*_case 64_(GenomeWideSNP_6).CEL	In	1.49	96.03	female	1	12/21/2014 10:18 AM
15	*H. pylori*_case 66_(GenomeWideSNP_6).CEL	Out	-0.06	82.40	Male	1	8/28/2014 4:08 PM
16	*H. pylori*_case 70_(GenomeWideSNP_6).CEL	In	1.96	96.62	female	1	12/21/2014 10:20 AM
17	*H. pylori*_case 71_(GenomeWideSNP_6).CEL	In	0.78	90.80	female	1	12/21/2014 10:21 AM
18	*H. pylori*_case 72_(GenomeWideSNP_6).CEL	In	0.99	93.78	Male	1	12/21/2014 10:22 AM
19	*H. pylori*_case 75_(GenomeWideSNP_6).CEL	Out	-0.17	85.11	female	1	12/21/2014 10:25 AM
20	*H. pylori*_case 76_(GenomeWideSNP_6).CEL	In	1.39	94.21	Male	1	12/21/2014 10:26 AM

Table 3.11 Continued

No	File	Bounds	Contrast QC	QC Call Rate	Computed Gender	# CHP/CEL	File Date
21	*H. pylori*_case81_(GenomeWideSNP_6).CEL	Out	0.07	87.33	male	1	12/21/2014 10:28 AM
22	*H. pylori*_case 83_(GenomeWideSNP_6).CEL	In	1.42	94.61	Female	1	12/21/2014 10:29 AM
23	*H. pylori*_case 85_(GenomeWideSNP_6).CEL	In	0.75	94.67	Female	1	12/21/2014 10:30 AM
24	*H. pylori*_case 86_(GenomeWideSNP_6).CEL	In	1.22	93.81	Female	1	12/21/2014 10:31 AM
25	*H. pylori*_case 90_(GenomeWideSNP_6).CEL	In	0.49	87.52	Male	1	11/4/2014 3:00 PM
26	*H. pylori*_case 91_(GenomeWideSNP_6).CEL	In	2.01	95.93	Female	1	12/21/2014 10:33 AM
27	*H. pylori*_case 92_(GenomeWideSNP_6).CEL	In	0.67	88.32	Female	1	11/4/2014 3:02 PM
28	*H. pylori*_case 97_(GenomeWideSNP_6).CEL	In	1.16	96.53	Male	1	12/21/2014 10:34 AM
29	*H. pylori*_case 100_(GenomeWideSNP_6).CEL	Out	0.00	84.75	Male	1	12/21/2014 2:36 PM
30	*H. pylori*_case 104_(GenomeWideSNP_6).CEL	In	1.05	88.85	Male	1	12/21/2014 2:37 PM
31	*H. pylori*_case 105_(GenomeWideSNP_6).CEL	In	0.65	87.52	Female	1	8/28/2014 4:09 PM
32	*H. pylori*_case 107_(GenomeWideSNP_6).CEL	In	1.99	95.96	Male	1	12/21/2014 9:13 AM
33	*H. pylori*_case 111_(GenomeWideSNP_6).CEL	In	1.85	94.41	Male	1	12/21/2014 9:17 AM
34	*H. pylori*_case 112_(GenomeWideSNP_6).CEL	In	2.03	96.00	Male	1	12/21/2014 2:34 PM
35	*H. pylori*_case 113_(GenomeWideSNP_6).CEL	In	0.57	91.96	Male	1	12/21/2014 9:18 AM
36	*H. pylori*_case 114_(GenomeWideSNP_6).CEL	In	1.09	95.67	Male	1	12/21/2014 9:19 AM
37	*H. pylori*_case 118_(GenomeWideSNP_6).CEL	In	1.49	94.74	Male	1	12/21/2014 9:23 AM
38	*H. pylori*_case 119_(GenomeWideSNP_6).CEL	In	1.28	93.22	Male	1	12/21/2014 9:24 AM
39	*H. pylori*_case 130_(GenomeWideSNP_6).CEL	In	1.04	90.64	female	1	12/21/2014 9:51 AM
40	*H. pylori*_case 133_(GenomeWideSNP_6).CEL	In	1.03	91.83	male	1	12/21/2014 9:52 AM

Table 3.11 Continued

No	File	Bounds	Contrast QC	QC Call Rate	Computed Gender	# CHP/CEL	File Date
41	*H. pylori*_case134_(GenomeWideSNP_6).CEL	In	1.43	94.67	male	1	12/21/2014 9:53 AM
42	*H. pylori*_case 141_(GenomeWideSNP_6).CEL	In	1.70	95.43	male	1	12/21/2014 9:55 AM
43	*H. pylori*_control 2_(GenomeWideSNP_6).CEL	In	0.92	91.53	male	1	9/4/2014 4:30 PM
44	*H. pylori*_control 4_(GenomeWideSNP_6).CEL	In	0.77	90.80	female	1	9/4/2014 4:32 PM
45	*H. pylori*_control 8_(GenomeWideSNP_6).CEL	In	1.41	97.29	female	1	12/21/2014 9:27 AM
46	*H. pylori*_control 12_(GenomeWideSNP_6).CEL	Out	-0.01	74.95	male	1	9/4/2014 4:29 PM
47	*H. pylori*_control 61_(GenomeWideSNP_6).CEL	In	1.64	96.39	male	1	12/21/2014 10:16 AM
48	*H. pylori*_control 62_(GenomeWideSNP_6).CEL	Out	-0.05	61.09	male	1	12/21/2014 2:35 PM
49	*H. pylori*_control 65_(GenomeWideSNP_6).CEL	In	1.94	96.10	male	1	12/21/2014 10:19 AM
50	*H. pylori*_control 74_(GenomeWideSNP_6).CEL	In	1.25	93.58	male	1	12/21/2014 10:23 AM
51	*H. pylori*_control 98_(GenomeWideSNP_6).CEL	In	1.31	94.61	male	1	12/21/2014 10:35 AM
52	*H. pylori*_control 99_(GenomeWideSNP_6).CEL	In	1.66	96.89	Female	1	12/21/2014 10:36 AM
53	*H. pylori*_control 102_(GenomeWideSNP_6).CEL	In	1.33	96.13	Male	1	12/21/2014 9:12 AM
54	*H. pylori*_control 108_(GenomeWideSNP_6).CEL	In	1.39	95.43	Male	1	12/21/2014 9:14 AM
55	*H. pylori*_control 109_(GenomeWideSNP_6).CEL	In	1.98	96.00	Male	1	12/21/2014 9:15 AM
56	*H. pylori*_control 110_(GenomeWideSNP_6).CEL	In	2.03	94.04	Female	1	12/21/2014 2:33 PM
57	*H. pylori*_control 115_(GenomeWideSNP_6).CEL	In	1.91	96.36	Male	1	12/21/2014 9:20 AM
58	*H. pylori*_control 116_(GenomeWideSNP_6).CEL	In	1.42	93.58	male	1	12/21/2014 9:21 AM
59	*H. pylori*_control 117_(GenomeWideSNP_6).CEL	In	2.14	96.89	male	1	12/21/2014 9:22 AM
60	*H. pylori*_control122_(GenomeWideSNP_6).CEL	In	1.76	94.57	male	1	12/21/2014 9:25 AM

Table 3.11 Continued

No	File	Bounds	Contrast QC	QC Call Rate	Computed Gender	# CHP/CEL	File Date
61	*H. pylori*_control 123_(GenomeWideSNP_6).CEL	In	0.86	87.72	male	1	12/21/2014 9:27 AM
62	*H. pylori*_control 124_(GenomeWideSNP_6).CEL	In	0.73	90.17	female	1	12/21/2014 9:28 AM
63	*H. pylori*_control 125_(GenomeWideSNP_6).CEL	In	0.57	93.78	Male	1	12/21/2014 9:29 AM
64	*H. pylori*_control 126_(GenomeWideSNP_6).CEL	In	1.30	93.61	Male	1	12/21/2014 9:30 AM
65	*H. pylori*_control 129_(GenomeWideSNP_6).CEL	In	2.28	97.95	Male	1	12/21/2014 9:49 AM
66	*H. pylori*_control 135_(GenomeWideSNP_6).CEL	In	1.91	95.96	female	1	12/21/2014 9:54 AM
67	*H. pylori*_control 144_(GenomeWideSNP_6).CEL	In	1.06	91.43	female	1	12/21/2014 9:56 AM
68	*H. pylori*_control 145_(GenomeWideSNP_6).CEL	In	1.73	96.16	Male	1	12/21/2014 9:57 AM
69	*H. pylori*_control 147_(GenomeWideSNP_6).CEL	In	1.65	95.23	Male	1	12/21/2014 9:58 AM
70	*H. pylori*_control 157_(GenomeWideSNP_6).CEL	In	1.77	94.57	Male	1	12/21/2014 2:39 PM
71	*H. pylori*_control 165_(GenomeWideSNP_6).CEL	In	1.34	94.97	female	1	12/21/2014 9:59 AM
72	*H. pylori*_control 166_(GenomeWideSNP_6).CEL	In	2.02	93.91	female	1	11/4/2014 2:58 PM
73	*H. pylori*_control 172_(GenomeWideSNP_6).CEL	Out	-0.20	89.54	Male	1	12/21/2014 10:00 AM
74	*H. pylori*_control 173_(GenomeWideSNP_6).CEL	In	1.22	95.83	Male	1	12/21/2014 10:01 AM
75	*H. pylori*_control 176_(GenomeWideSNP_6).CEL	In	1.96	94.80	Male	1	12/21/2014 10:02 AM
76	*H. pylori*_control 198_(GenomeWideSNP_6).CEL	Out	0.28	87.72	Male	1	11/4/2014 2:59 PM
77	*H. pylori*_control 199_(GenomeWideSNP_6).CEL	In	2.18	94.80	female	1	12/21/2014 2:40 PM
78	*H. pylori*_control 200_(GenomeWideSNP_6).CEL	In	2.53	97.58	Male	1	12/21/2014 2:41 PM
79	*H. pylori*_control 201_(GenomeWideSNP_6).CEL	In	1.81	96.33	Male	1	12/21/2014 2:42 PM
80	*H.pylori*_control 203_(GenomeWideSNP_6).CEL	In	1.69	94.51	female	1	12/21/2014 10:04 AM

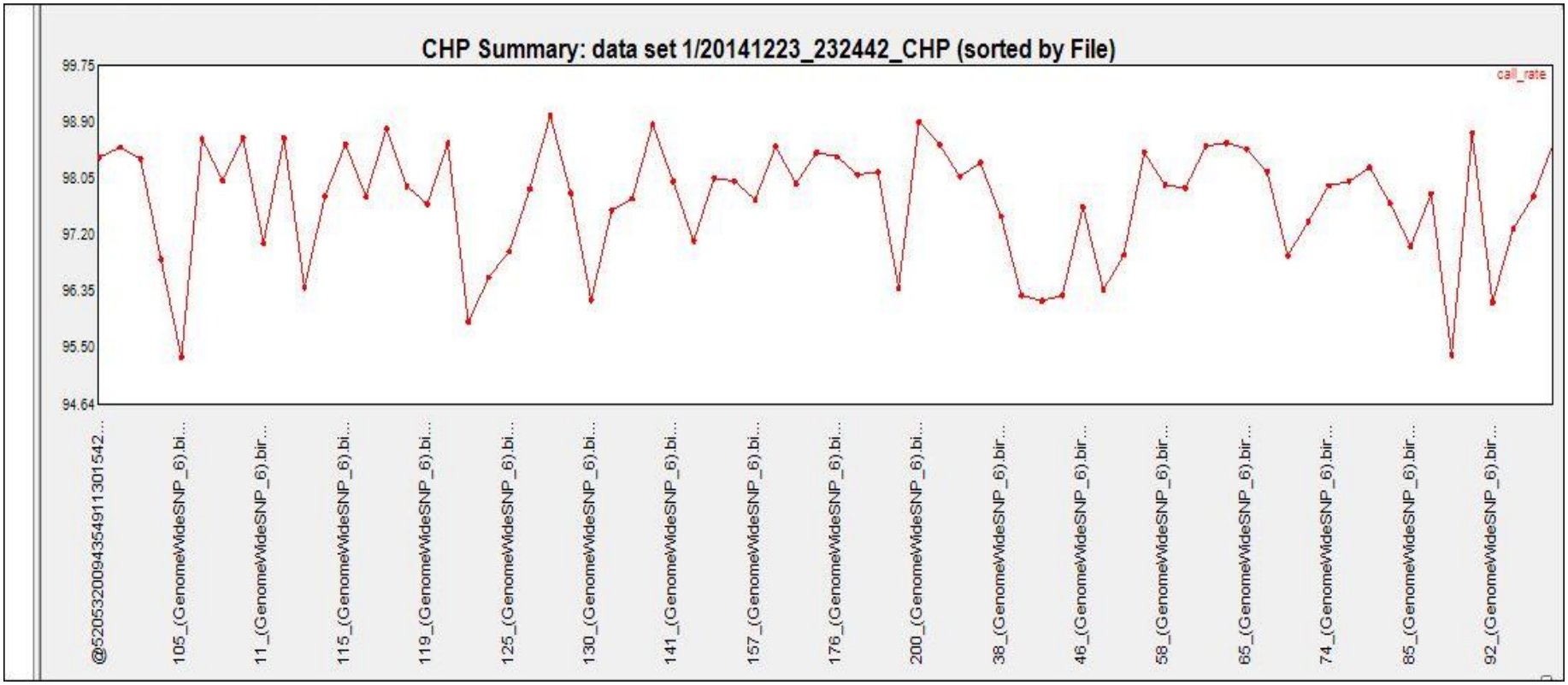

Figure 3.15 Line graph showing intensity QC generated using GTC software

The Y-axis shows QC call rate; the X-axis shows *H. pylori* samples

3.2.3 *H. pylori* positive patients and phenotype association analysis

Out of the total 42 *H. pylori* positive patients recruited in this study, 4 samples were excluded from further analysis due to low QC call rate . The rest of 38 *H. pylori* patients were group into gastritis and peptic ulcer disease based on the endoscopic findings. Majority of the patients had gastritis (34) while only four patients had peptic ulcer disease (Table 3.12). The Indians had 20 cases, whilst Malays and Chinese had 9 cases each.

Genotype-phenotype association analysis was done by using Plink (version 1.07) software. The Plink (version 1.07) software generated spreadsheet for each genotype under study. These results were used to create Manhattan plot for each gastritis and peptic ulcer disease under each ethnic group (Figures 3.16, 3.17, 3.18, 3.19 and 3.20). The top SNPs from Manhattan plot results in each phenotype under different ethnic group is summarized in Table 3.13, 3.14, 3.15, 3.16 and 3.17.

Table 3.12 *H. pylori* infected patients and associated phenotype

No	Case	Phenotype	Ethnicity
1	*H. pylori*_case5	Gastritis	Chinese
2	*H. pylori*_case 11	Gastritis	Malay
3	*H. pylori*_case 13	Gastritis	Malay
4	*H. pylori*_case 19	Gastritis	Indian
5	*H. pylori*_case 21	Gastritis	Indian
6	*H. pylori*_case 38	Gastritis	Indian
7	*H. pylori*_case 43	Gastritis	Chinese
8	*H. pylori*_case 45	Gastritis	Indian
9	*H. pylori*_case 46	Gastritis	Malay
10	*H. pylori*_case 52	Gastritis	Indian
11	*H. pylori*_case 53	Gastritis	Indian
12	*H. pylori*_case 58	Gastritis	Indian
13	*H. pylori*_case 59	Gastritis	Indian
14	*H. pylori*_case 64	Gastritis	Indian
15	*H. pylori*_case 70	Peptic ulcer	Chinese
16	*H. pylori*_case 71	Gastritis	Indian
17	*H. pylori*_case 72	Peptic ulcer	Indian
18	*H. pylori*_case 76	Gastritis	Chinese
19	*H. pylori*_case 83	Gastritis	Indian
20	*H. pylori*_case 85	Gastritis	Indian
21	*H. pylori*_case 86	Gastritis	Malay
22	*H. pylori*_case 90	Gastritis	Malay
23	*H. pylori*_case 91	Gastritis	Chinese
24	*H. pylori*_case 92	Gastritis	Malay
25	*H. pylori*_case 97	Gastritis	Indian

Table 3.12 continued

No	Case	Phenotype	Ethnicity
26	*H. pylori*_case 104	Gastritis	Malay
27	*H. pylori*_case 105	Gastritis	Indian
28	*H. pylori*_case 107	Gastritis	Chinese
29	*H. pylori*_case 111	Gastritis	Chinese
30	*H. pylori*_case 112	Peptic ulcer	Chinese
31	*H. pylori*_case 113	Peptic ulcer	Indian
32	*H. pylori*_case 114	Gastritis	Malay
33	*H. pylori*_case 118	Gastritis	Malay
34	*H. pylori*_case 119	Gastritis	Indian
35	*H. pylori*_case 130	Gastritis	Indian
36	*H. pylori*_case 133	Gastritis	Indian
37	*H. pylori*_case134	Gastritis	Indian
38	*H. pylori*_case 141	Gastritis	Chinese

3.2.3.1 SNPs associated with *H. pylori* gastritis and peptic ulcer phenotype among Indian population

In this study, a total of (18/38) *H. pylori* infected patients were diagnosed as having gastritis. SNPs analysis was conducted by using Plink (version 1.07) software which generated a total of 898, 725 SNPs. Out of this only 690, 885 SNPs were included in the final association analysis. The rest of SNPs were eliminated by SNP filtering parameters; those having a minor allele frequency (MAF) > 0.01, those deviating from Hardy-Weinberg equilibrium HWE p-value > 0.001 and SNP call rate > 95%. Figure 3.16 shows Manhattan plot of 690, 885 SNPs. The most two significant SNPs are rs1809578 and rs3770521 with p-value of $9.85x\ 10^{-6}$ and $1.33\ x^{-5}$ respectively (Figure 3.16 and Table 3.13). rs1809578 (P=9.85x 10 -6) which is located in chromosome 4 and associated with BANK1 gene (B-cell scaffold protein with ankyrin repeats 1) attained the most protective SNPs. SNPs rs3770521 (P=1.33 x-5) is the most susceptible SNP in *H. pylori* gastritis among the Indian groups. This SNP is located in chromosome 2 and associated with XRCC5 gene (Human X-ray repair crosscomplementing 5). The position of rs3770521 in gene XRCC5 was shown in Figure 3.21. Other top most SNPs were shown in Table 3.13 while the rest are in Appendix AI.

H. pylori peptic ulcer phenotype was found in (2/38) among the Indian Population. SNPs analysis was done by using Plink (version 1.07) software which generated a total of 898, 725 SNPs. Out of this only 615, 677 SNPs were included in the final association analysis. The rest of SNPs were eliminated by SNP filtering parameters; those having MAF > 0.01, those deviating from HWE p-value > 0.001 and SNP call rate > 95%. Figure 3.17 shows Manhattan plot of 615, 677 SNPs. The

most top SNP is rs7725568 with p-value of 0.3889. SNP rs7725568 is located in chromosome 5 and associated with gene MAST4 (microtubule associated serine/threonine kinase family member 4) (Table 3.14). Other top SNPs are shown in Table 3.14.

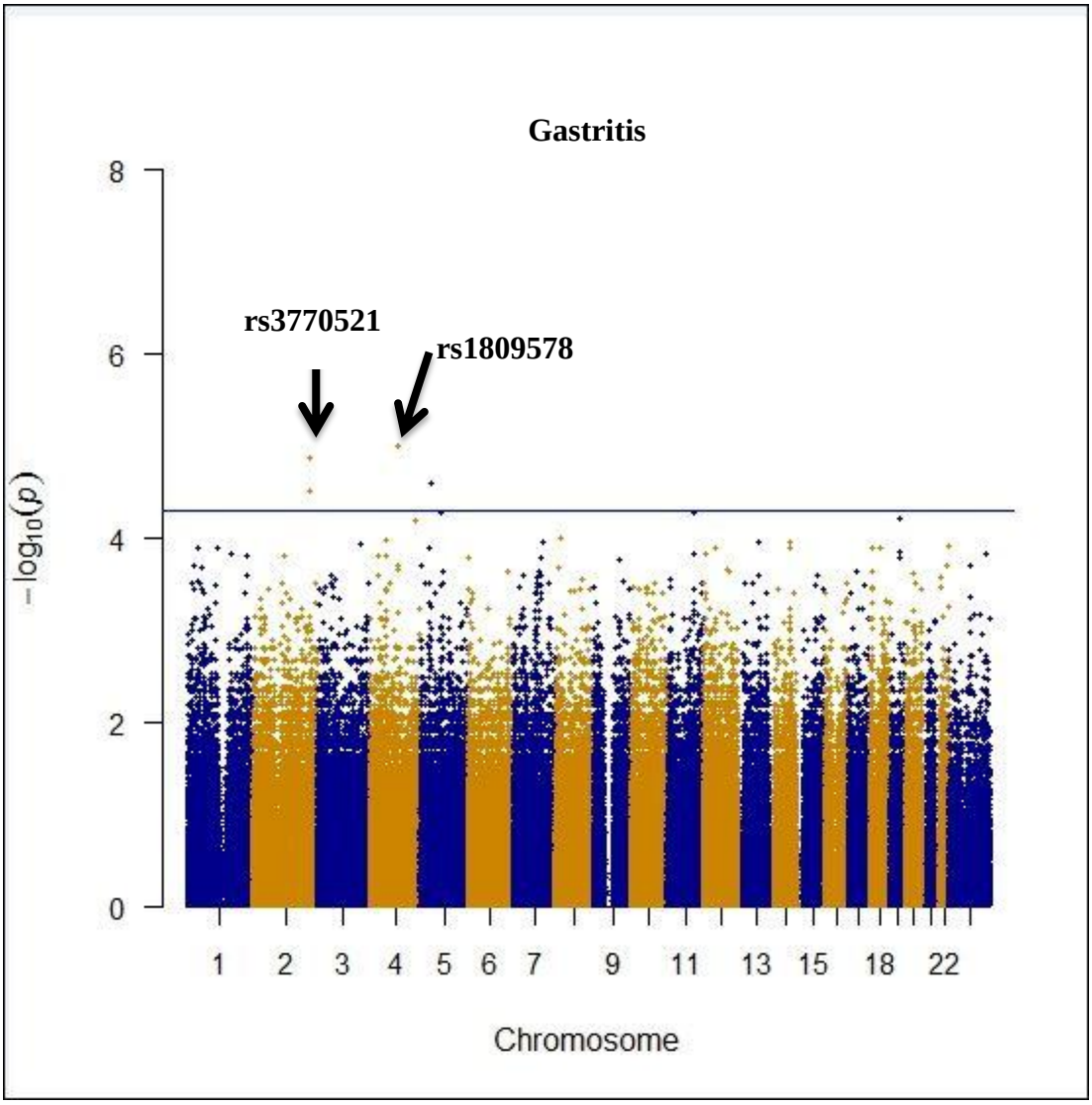

Figure 3.16 Manhattan plot of genotype association test for Indian gastritis patients.

The plot shows $-\log_{10}$ P values for each SNP against chromosomal location. The X-axis shows chromosomal positions and Y-axis shows $-\log_{10}$ P-values. The blue line indicates the SNP of P- value of 1.0×10^{-5}.

Table 3.13 Top SNPs from the genome-wide association study associated with *H. pylori* gastritis phenotype in Indians

No	CHR	dbSNP ID	BP	Gene	Minor allele	MAF - cases	MAF - control	P-value
1	4	rs1809578	102852575	BANK1	T	0.3824	0.8889	**9.85E-06**
2	2	rs3770521	216980160	XRCC5	A	0.4722	0.02778	**1.33E-05**
3	5	rs6523782	43258528	IL1RAPL2	A	0.1111	0.5833	2.57E-05
4	2	rs10182201	216954572	TMEM169	G	0.4444	0.02778	3.15E-05
5	5	rs557302	78377334	BHMT2	G	0.6667	0.1944	5.21E-05
6	11	rs2846724	102569503	MMP27	C	0.6667	0.1944	5.21E-05
7	19	rs741587	34167905	CHST8	A	0.05556	0.4722	6.05E-05
8	4	rs17060468	175249433	CEP44	A	0.4444	0.8889	6.33E-05
9	8	rs4921941	18459588	PSD3	G	0.5	0.08333	0.000101
10	4	rs6822974	63006443	LPHN3-AS1	G	0.3333	0.7941	0.000105

CHR, Chromosome; bp, base pair; MAF-case, minor allele frequency in cases; MAF-control, minor allele in controls

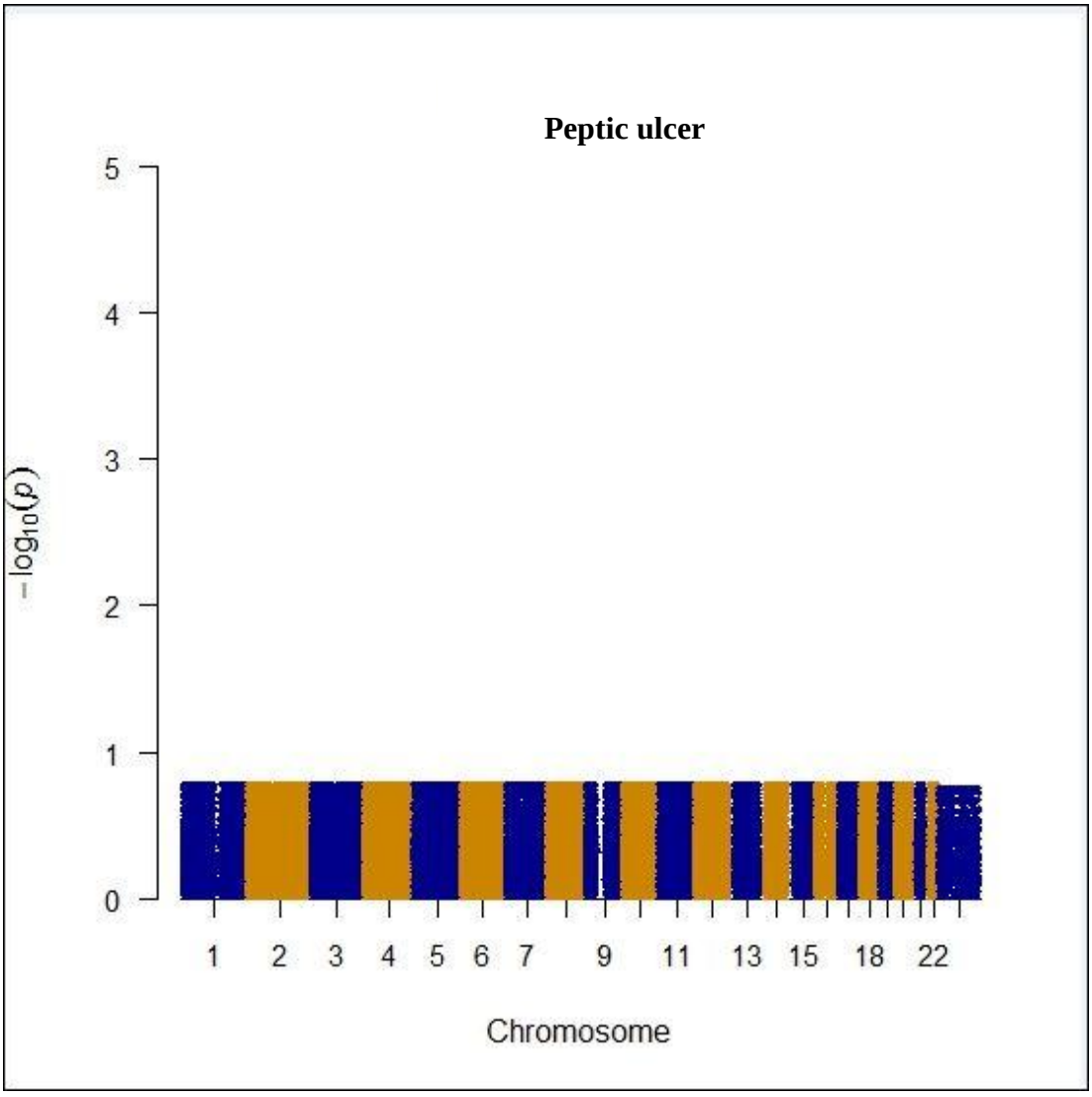

Figure 3.17 Manhattan plot of genotype association test for Indian Peptic ulcer disease patients.

The plot shows –log10 P values for each SNP against chromosomal location. The X-axis shows chromosomal positions and Y-axis shows –log10 P-values.

Table 3.14 Top SNPs from the genome-wide association study associated with *H. pylori* Peptic ulcer phenotype in Indians

No	CHR	dbSNP ID	BP	Gene	Minor allele	MAF-case	MAF-control	P-value
1	5	rs7725568	66115982	MAST4	C	0.75	0.3889	**0.1657**
2	5	rs173651	66316009	MAST4	G	0.75	0.3889	0.1657
3	5	rs2931426	73209251	ARHGEF28	A	0.25	0.6111	0.1657
4	5	rs3846665	74681500	COL4A3BP	C	0.25	0.6111	0.1657
5	5	rs6861279	74919409	ANKDD1B	A	0.75	0.3889	0.1657

CHR, chromosome; bp, base pair; MAF-case, minor allele frequency in cases; MAF-control, minor allele in controls

3.2.3.2 SNPs associated with *H. pylori* gastritis phenotype among Malay population

In this study, a total of (9/38) *H. pylori* infected patients were identified with *H. pylori* gastritis. SNPs analysis was conducted by using Plink (version 1.07) software and it generated a total of 898, 725 SNPs. Out of these only 624, 048 SNPs were included in the final association analysis. The rest of SNPs were eliminated by SNP filtering parameters; those having MAF > 0.01, those deviating from HWE p-value > 0.001 and SNP call rate > 95%. Figure 3.18 shows Manhattan plot of 624, 048 SNPs. The most two significant SNP were rs7042986 and rs3776349 with p-value of 0.0001 and 0.0001 respectively (Figure 3.18 and Table 3.15). rs7042986 which was located in chromosome 9 and associated with gene SMARCA2 (SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 2) attained the most signifant SNPs (P=.0.0001). The position of SNP rs7042986 of gene SMARCA2 was shown in Figure 3.22. SNPs rs3776349 (P=0.0001) is located in chromosome 5 and associated with gene ARHGAP26 (Rho GTPase activating protein 26). Other top most SNPs are shown in Table 3.15 while the rest are in Appendix AII.

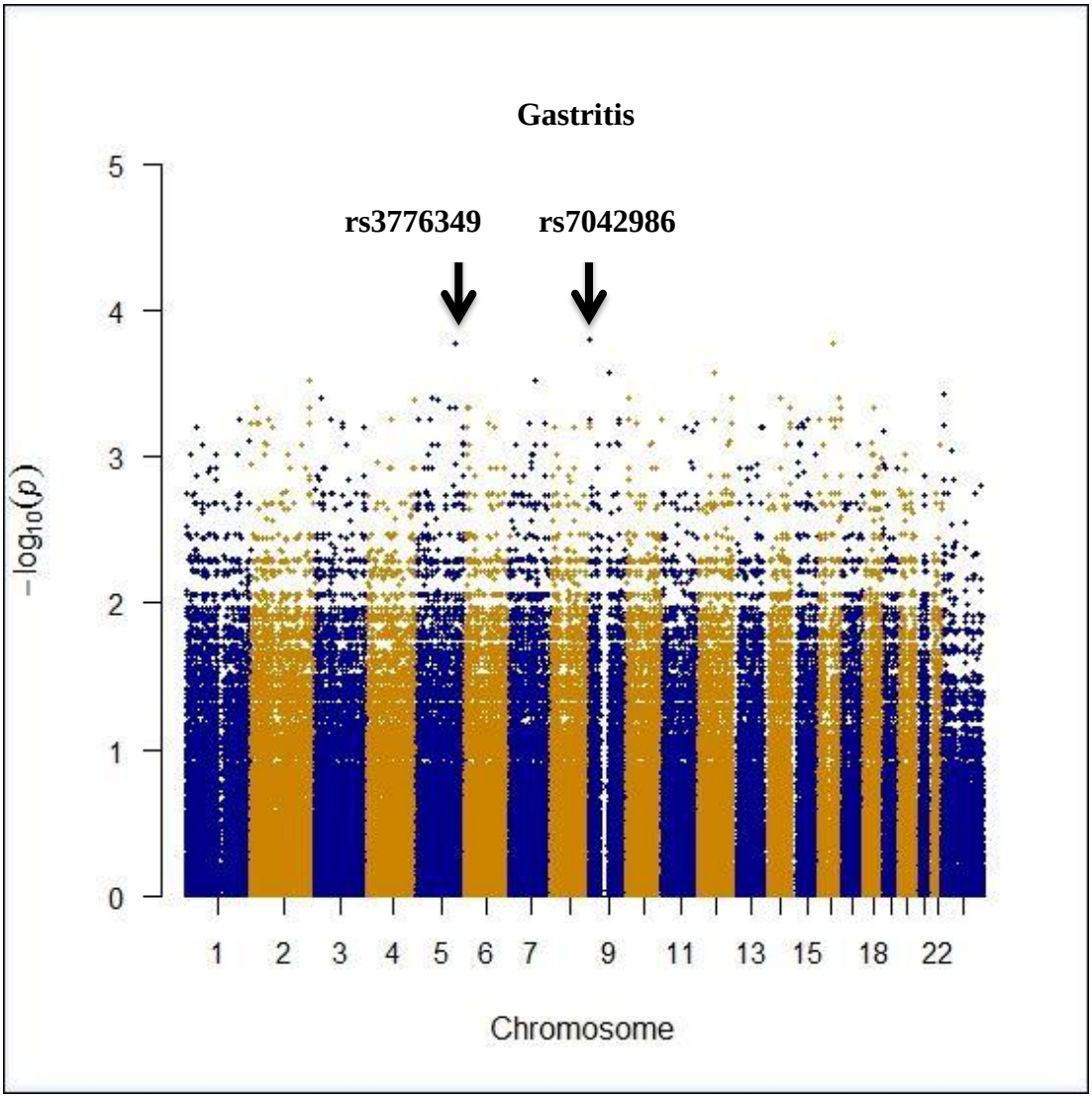

Figure 3.18 Manhattan plot of genotype association test for Malay gastritis patients.

The plot shows $-\log_{10}$ P values for each SNP against chromosomal location. The X-axis shows chromosomal positions and Y-axis shows $-\log_{10}$ P-values.

Table 3.15 Top SNPs from the genome-wide association study associated with *H. pylori* gastritis phenotype in Malay

No	CHR	dbSNP ID	BP	Gene	Minor allele	MAF-cases	MAF-control	P-value
1	9	rs7042986	2184258	SMARCA2	T	0.8889	0.25	**0.0001**
2	5	rs3776349	142430200	ARHGAP26	G	0.1667	0.8125	**0.0001**
3	16	rs1965229	57721146	GPR97	G	0.1667	0.8125	0.000166
4	12	rs2706309	60127590	SLC16A7	T	0.0625	0.6875	0.000261
5	9	rs2498430	71684702	FXN	G	0.6875	0.0625	0.000261
6	2	rs7571732	223538316	MOGAT1	C	0.3333	0.9375	0.000297
7	7	rs854569	94950055	PON1	G	0.3333	0.9375	0.000297
8	23	rs5934677	6868042	LOC101928246	T	0.1667	0.9091	0.00037
9	3	rs1603663	22036489	ZNF385D	G	0.05556	0.625	0.0003961
10	5	rs295669	58147456	RAB3C	C	0.05556	0.625	0.0003961

CHR, chromosome; bp, base pair; MAF-case, minor allele frequency in cases; MAF-control, minor allele in controls

3.2.3.3 SNPs associated with *H. pylori* gastritis and peptic ulcer phenotype among Chinese population

In this study, a total of 7 out of 38 *H. pylori* infected patients were identified as having gastritis. SNPs analysis was done by using Plink (version 1.07) software which generated a total of 898, 725 SNPs. Out of this only 559, 185 SNPs were included in the final association analysis. The rest of SNPs were eliminated by SNP filtering parameters; those having a minor allele frequency (MAF) > 0.01, those deviating from Hardy-Weinberg equilibrium HWE p-value > 0.001 and SNP call rate > 95%. Figure 3.19 shows Manhattan plot of 559, 185 SNPs. The most top significant SNP were rs10860808 with same p-value of 0.0002 (Figure 3.19 and Table 3.16). SNP rs10860808 was located in chromosome 12 and this SNP is associated with gene DRAM1 (DNA-damage regulated autophagy modulator 1). The position of SNP rs10860808 was shown in Figure 3.23. Other top most SNPs are shown in Table 3.16 while the rest are in Appendix AIII.

H. pylori peptic ulcer phenotype was found in 2 out of 38 the Chinese Population. SNPs analysis was done by using Plink (version 1.07) software which generated a total of 898, 725 SNPs. Out of this only 571, 530 SNPs were included in the final association analysis. The rest of SNPs were eliminated by SNP filtering parameters; those having MAF > 0.01, those deviating from HWE p-value > 0.001 and SNP call rate > 95%. Figure 3.20 shows Manhattan plot of 571, 530 SNPs. The most top SNP is rs8061985 with p-value of 0.06076. SNP rs8061985 which is located in chromosome 16 and associated with gene CDH13 (cadherin 13) (Table 3.17). Other top SNPs are shown in Table 3.17.

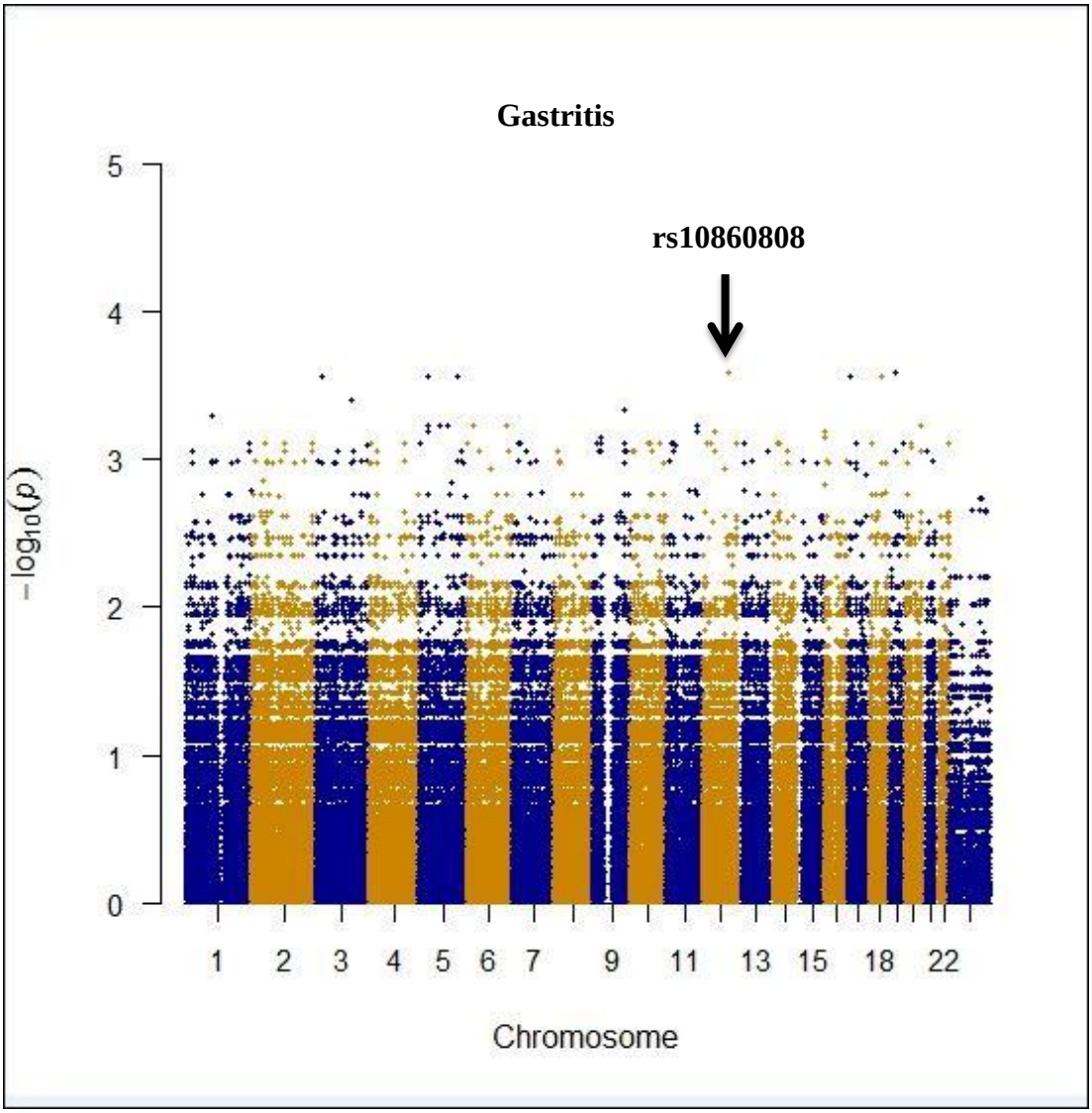

Figure 3.19 Manhattan plot of genotype association test for Chinese gastritis patients. The plot shows $-\log_{10}$ P values for each SNP against chromosomal location. The X-axis shows chromosomal positions and Y-axis shows $-\log_{10}$ P-values.

Table 3.16 Top SNPs from the genome-wide association study associated with *H. pylori* gastritis phenotype in Chinese

No	CHR	dbSNP ID	BP	Gene	Minor allele	MAF-cases	MAF-control	P-value
1	12	rs10860808	102272659	DRAM1	A	0.8571	0.1875	**0.0002**
2	19	rs1216115	22709636	LOC101929124	A	0.1429	0.8125	0.000253
3	3	rs7611730	19449092	KCNH8	C	0.2143	0.875	0.0002691
4	5	rs3844310	34819280	RAI14	C	0.2143	0.875	0.0002691
5	5	rs7722035	142055973	FGF1	C	0.2143	0.875	0.0002691
6	17	rs356054	6443244	PITPNM3	G	0.2143	0.875	0.0002691
7	17	rs356053	6444676	PITPNM3	T	0.2143	0.875	0.0002691
8	18	rs3893060	44309781	ST8SIA5	T	0.2143	0.875	0.0002691
9	3	rs10935257	137336749	intergenic	G	0.1	0.8125	0.0003919
10	9	rs2900570	116710992	ZNF618	G	0.08333	0.75	0.0004645

CHR, chromosome; bp, base pair; MAF-case, minor allele frequency in cases; MAF-control, minor allele in controls

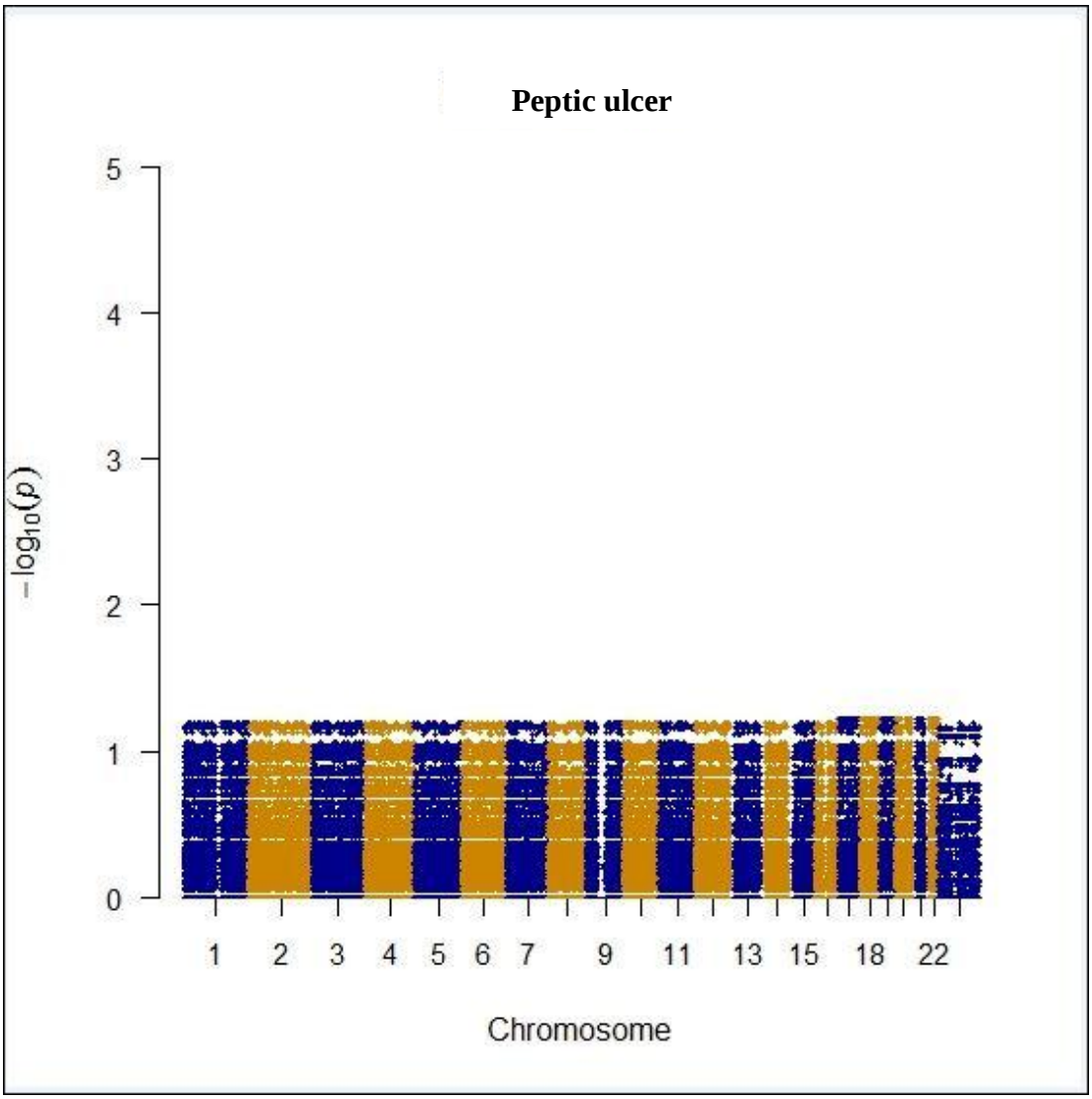

Figure 3.20 Manhattan plot of genotype association test for Chinese peptic ulcer patients.

The plot shows –log10 P values for each SNP against chromosomal location. The X-axis shows chromosomal positions and Y-axis shows –log10 P-values.

Table 3.17 Top SNPs from the genome-wide association study associated with *H. pylori* Peptic ulcer phenotype in Chinese

No	CHR	dbSNP ID	BP	Gene	Minor allele	MAF-cases	MAF-control	P-value
1	16	rs8061985	83109108	CDH13	C	0.25	0.75	0.06076
2	17	rs9894837	441275	VPS53	A	0.25	0.75	0.06076
3	17	rs150857	3526637	SHPK	C	0.25	0.75	0.06076
4	17	rs11869897	10245685	MYH13	T	0.25	0.75	0.06076
5	17	rs12325711	11259037	SHISA6	G	0.25	0.75	0.06076

bp, base pair; MAF-case, minor allele frequency in cases; MAF-control, minor allele in controls

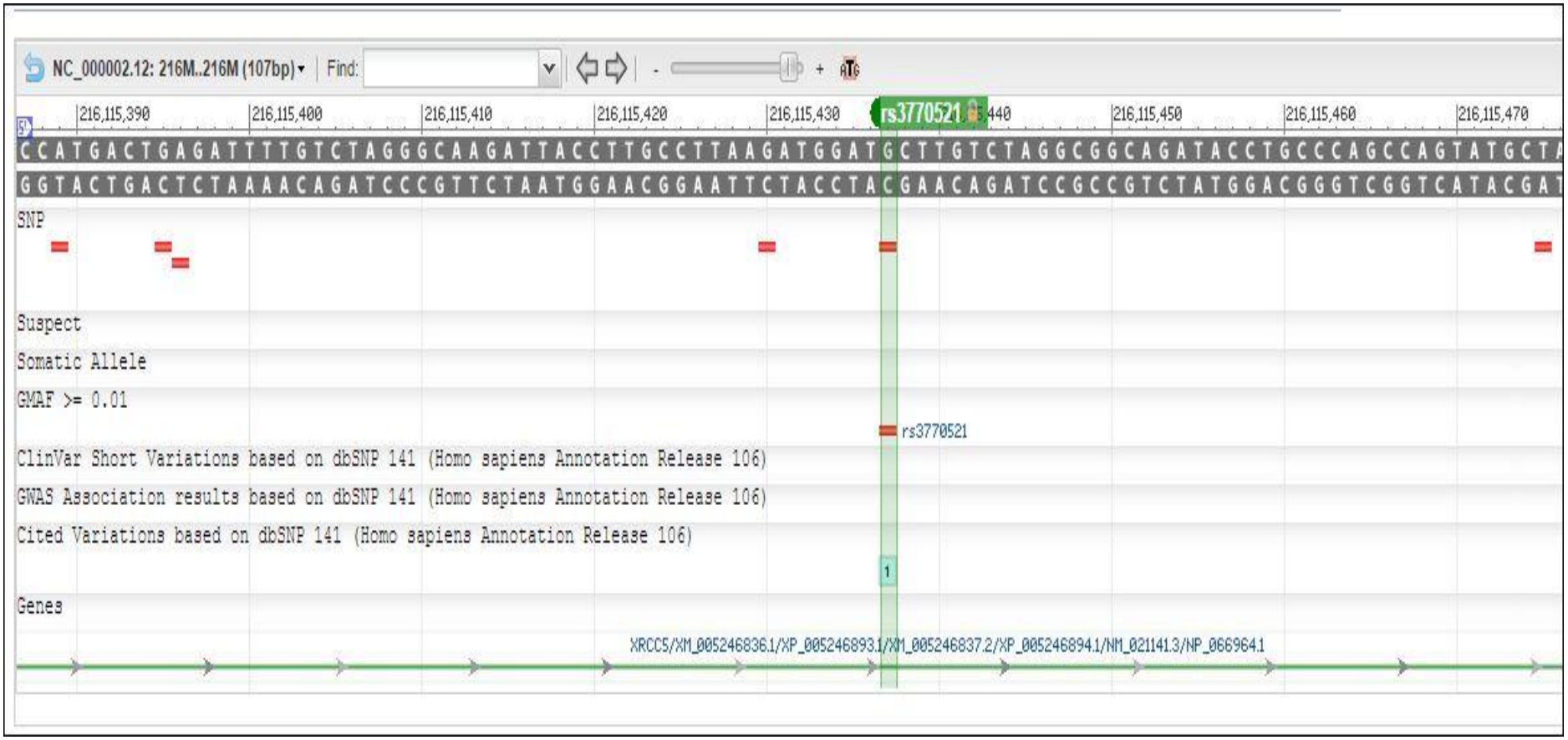

Figure 3.21 Position of SNP rs3770521 of gene XRCC5 (http://www.ncbi.nlm.nih.gov/)

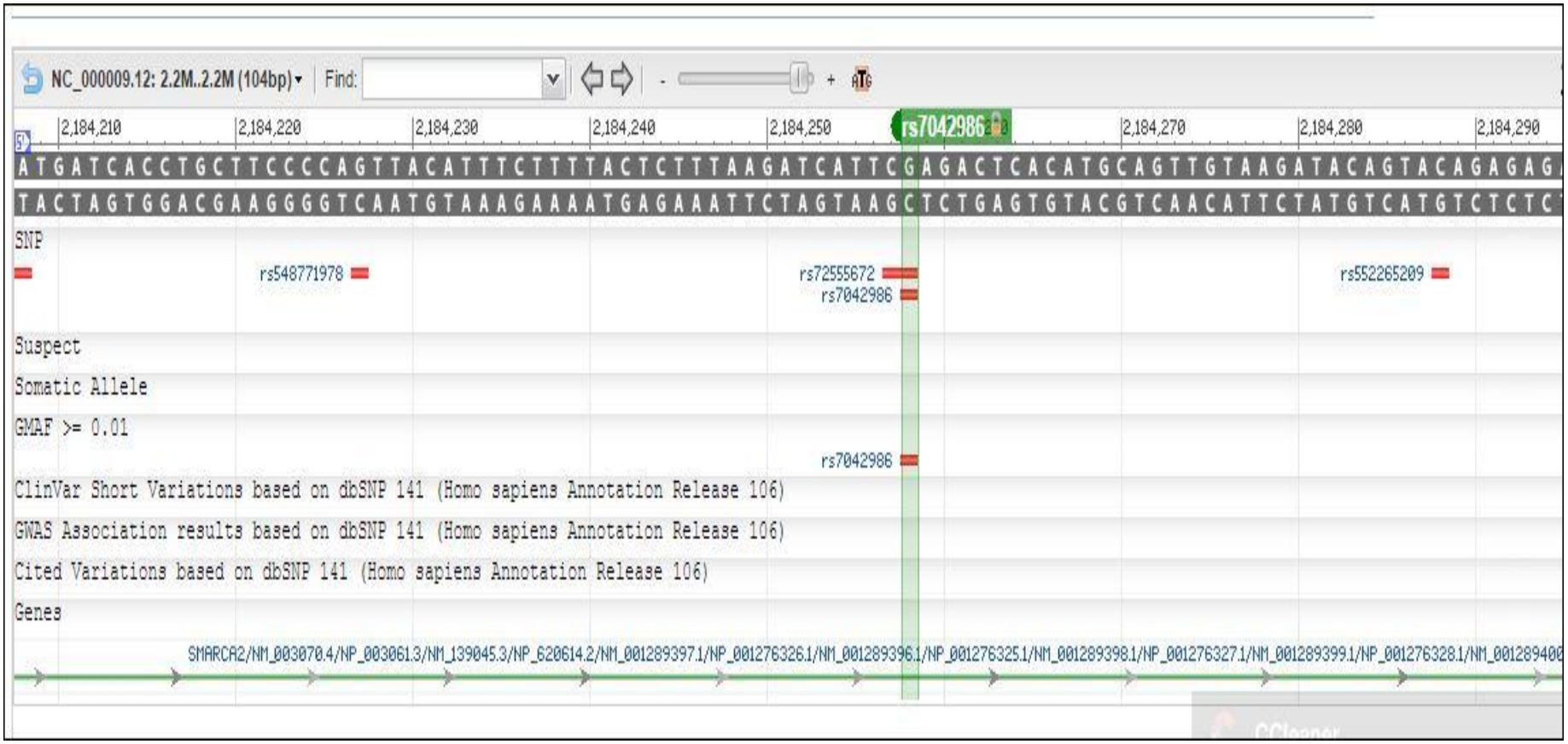

Figure 3.22 Position of SNP rs7042986 of gene SMARCA2 (http://www.ncbi.nlm.nih.gov/)

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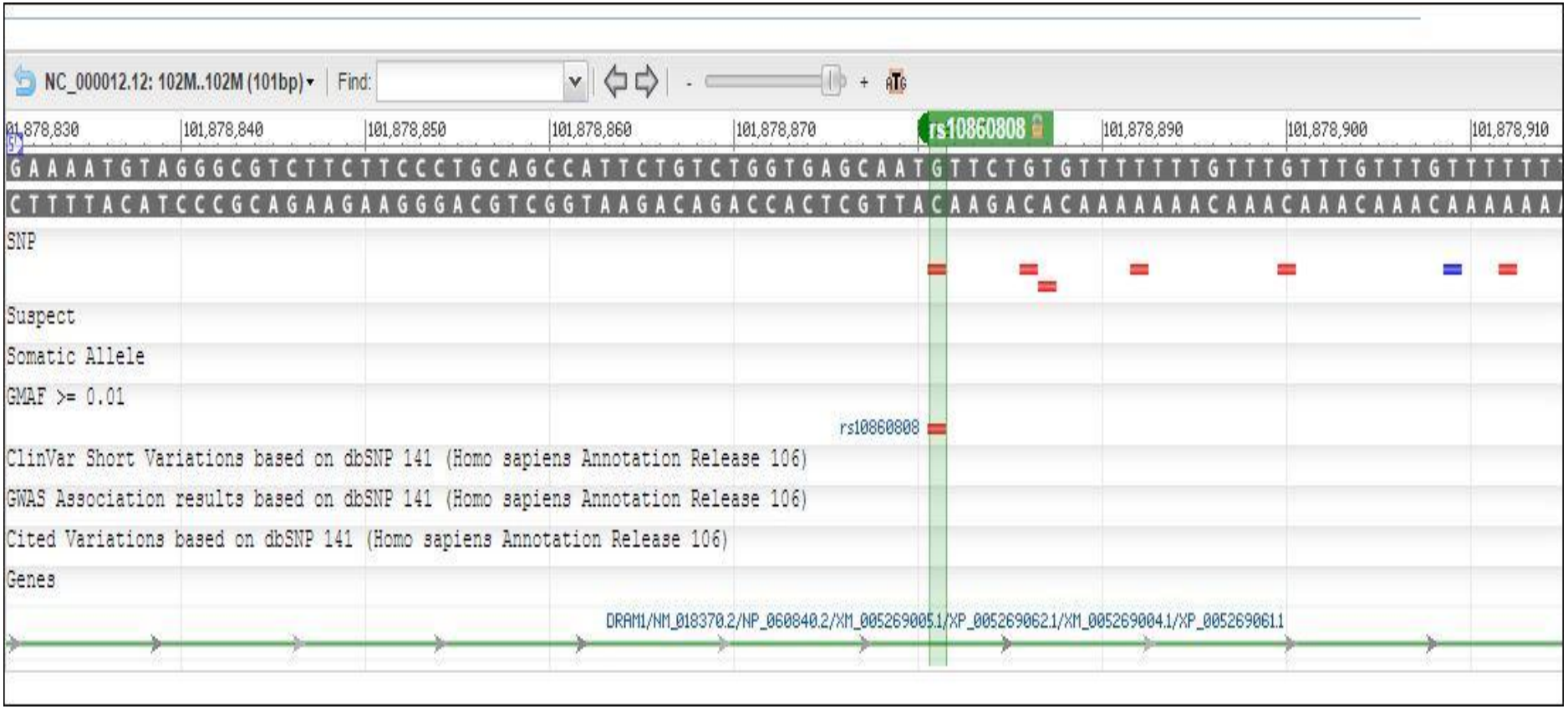

Figure 3.23 Position of SNP rs10860808 of gene DRAM1 (http://www.ncbi.nlm.nih.gov/)

Chapter 4: Discussion

4.1 Phase I: *H. pylori cagA*, *dupA*, *babA*2 and *SabA* and *cagA* EPIYA motifs

4.1.1 Clinical outcome, age and race in *H. pylori* infected patients

H. pylori is a Gram-negative, spiral shaped bacterium that infects the stomach of approximately half of the world's human population (Kusters *et al.*, 2006)). It is acquired early in life and remains for the lifetime in the host. Infection is frequently linked with the development of chronic gastritis, peptic ulcers and gastric cancer (Fock and Ang, 2010). *H. pylori* infection is associated with factors such as age, socioeconomic status, childhood crowding, and ethnicity. Therefore, information about specific risk groups may allow assessment of disease risk and will offer chances for targeted interventions (Goh *et al.*, 2011).

The comparison of the present distribution of patients with others mentioned in the literature highlights shows similarity as well as differences. In the present study, out of 105 patients who were positive for *H. pylori*, 74.3% had gastritis, 8.6% had DU, 4.7% had GU and 12.4% had normal endoscopic findings. This corroborates the findings of Ramellah *et al*. who reported a prevalence of 69.27% gastritis, 6.83% duodenal ulcer and 16.59% gastric ulcer in *H. pylori* infected patients while in different studies they found the distribution of gastritis 47.27%, duodenal ulcer 9.09% and gastric ulcer 13.64% (Ramelah *et al.*, 2005; Mohamed *et al.*, 2009).

A study done in Brazil also showed high number of gastritis patients 41.90% as compared with duodenal ulcer 22.44%, gastric ulcer 18.95% and gastric cancer

16.71% (Vinagre *et al.*, 2013). In another study done in Cambodia, they reported that 90.91% of *H. pylori* infected patients had gastritis (Breurec *et al.*, 2011b). The current study is in disagreement with study done in India which found that 66.67% of patient had PUD while 33.33% had gastritis (Tiwari *et al.*, 2005). Thus it appears that the distribution of diseases varied and might depend on the selection of patients, virulence of the infecting *H. pylori* strains, the type and the extent of the host immune response to infection (Atherton, 2006).

The present study indicated the highest occurrence of *H. pylori* infection among patients suffering from gastritis with ages 44-65 years. Moreover the highest infected groups were age group 44-65. It is believed that *H. pylori* infection is strongly related to the historically high rate of infection acquired in childhood, but disease manifestations typically do not appear until adulthood and often only after long periods of latency. This study is similar to the study done by Nouraie *et al.* which found the most infected age group was between 46-55 years (Nouraie *et al.*, 2009). DU and GU were found more common among the older ages, which might give an indication that this bacterium needs more time to induce peptic ulcer (Suerbaum and Josenhans, 2007).

In this study, the distribution of *H. pylori* among males (54.3%) is slightly higher as compared with females. This study is in agreement with a meta analysis conducted on relationship between gender and *H. pylori* in United States of America which reported that the male gender is significantly associated with *H. pylori* (de Martel and Parsonnet, 2006). The reason for the possible gender difference is undistinguishable but may relate to social gender role where men do outdoor activities more than women, which brings more risks of infection. Furthermore, men

take part in more risky behaviours such as smoking, alcohol drinking than women (Ozaydin *et al.*, 2013).

Other studies have found that the female gender has more risk. A study in Netherlands found that lower socioeconomic status and lack of antibiotic use were associated with female gender (den Hollander *et al.*, 2014). Otters *et al.* also reported that boys of up to 4 years of age received more antibiotics than girls of the same age (Otters *et al.*, 2004).

Malaysia is a multicultural country comprising of three major ethnic groups: Malays, Chinese and Indians. The Malays are the majority, the Malaysian-Chinese comprise the second largest ethnic group and are believed to have migrated from Southern China while the Malaysian-Indian group is comprised of migrants from Southern India (Kumar *et al.*, 2015). Previous studies have reported high prevalence of *H. pylori* infection among Indians, followed by Chinese and Malays with the lowest (Tan *et al.*, 2005; Tay *et al.*, 2009).

The present study showed that the distribution of *H. pylori* varied among different ethnicities. Malay has the highest infection (40.0%); Indians (35.2%) while Chinese have lowest infection rate (24.8%). The reason as to why the Malays have a higher prevalence rate may be due to the large population of the Malays in the current study. The difference among different ethnic groups is similar to a study on iron deficiency in New Zealand which showed difference in *H. pylori* prevalence according to ethnicity. They found that Pacific Island students have the highest followed by Maori and Asian students and lowest in the European students (Fraser *et al.*, 2010).

A multi-ethnic population study done in Netherland among Dutch women and non-Dutch ethnicity observed that ethnicity is the strongest predictor of *H. pylori* colonization in young women. *H. pylori* prevalence in Dutch women was 24%, which was significantly lower than in non-Dutch women (64%; $p<0.001$). *H. pylori* positivity was found in 92% of Moroccan, 80% of Cape Verdean, 81% of Turkish, 60% of Dutch Antillean and 58% of Surinamese women (den Hollander *et al.*, 2013)

In this study, there was a general trend in which those with higher education have a lower prevalence of infection. These results were consistent with previous studies (Shi *et al.*, 2008; Benajah *et al.*, 2013), signifying that education level may be an independent risk factor of *H. pylori* infection.

4.1.2 Detection of *H. pylori* by Atlas *H. pylori* antigen test

H. pylori is acquired in childhood and survives in the human stomach (Tan and Wong, 2011; Valliani *et al.*, 2013). Noninvasive testing for *H. pylori* has been strongly recommended as it is less expensive and more patient-friendly than invasive testing that requires endoscopy (Manes *et al.*, 2001).

The new stool antigen test showed high sensitivity (91.7%) and specificity (100%). This result is in agreement with other studies done on monoclonal stool antigen test based on immune chromatography. A study done in Brazil, found the sensitivity and specificity of monoclonal stool antigen test as 88.0% and 87.5%, respectively (Silva *et al.*, 2010). Similarly a study done in Turkey revealed the sensitivity and specificity of HpSA test as 68.9% and 100% respectively (Ceken *et*

al., 2011). Furthermore, a study done in Korea, found the sensitivity and specificity of *H. pylori* stool antigen immunochromatographic assay (S-ICT test) as 84.5% and 96.2% respectively (Jekarl *et al.*, 2013).

The advantages of Atlas *H. pylori* antigen test over stool antigen test especially HpSA test which has been validated and widely used is that it is easy and the test takes less than 10 min. HpSA test which is based on enzyme immunoassay tests takes over 1 hour to avail the result, Therefore the new Atlas *H. pylori* antigen test is more convenient and saves patient time. The Atlas *H. pylori* antigen test is a new non-invasive method which is simple to perform and avails the result in few minutes. This results have shown that, it has high sensitivity, specificity and diagnostic accuracy and can be used as an alternative method in the diagnosis of *H. pylori* infection in adults. However, there is a need for further studies with a greater number of different patients and to find also its effectiveness in the post treatment setting.

4.1.3 Test agreement among culture, RUT and Atlas *H. pylori* stool antigen test

Detection of *H. pylori* infection on gastric biopsy specimens usually uses RUT because the results can be interpreted easily, fast and can provide a result before patient is discharged from the endoscope room (Lim *et al.*, 2004). In the current study, the agreement between RUT and culture was very good and statistically significant ($k = 0.746$ and $P \leq 0.0001$). This is in agreement with other study which found strong agreement (Chomvarin *et al.*, 2006). To the best of our knowledge, this is the first study that evaluated Atlas *H. pylori* stool antigen test for detection of *H. pylori* infection in stool. The current study found very

good agreement between Atlas *H. pylori* stool antigen test and culture (k = 0.814 and P ≤ 0.0001). Other study that evaluated the use of similar test like HpSA test also found similar result (Falsafi *et al.*, 2005).

4.1.4 Distribution of *H. pylori cagA, babA2, dupA* and *sabA* in ethnic groups

The prevalence of *H. pylori* varies among the different ethnic groups. Furthermore this difference is also reflected in *H. pylori* strains variation among different ethnic groups living in the same area (Dabiri *et al.*, 2009; Amjad *et al.*, 2010). In the current study, the distribution of *cagA* is high among Indian population and lowest in the Chinese ethnic groups. *H. pylori babA2* and *SabA* are are predominant in Malay population and lowest among the Chinese population. In addition, *H. pylori dupA* has equal distribution among Indian and Malay population but lowest in Chinese ethnic groups.

The current study displays the distinctive difference in *H. pylori* genotypes in three ethnic groups residing within the same city. This is consistent with another study from Houston, Texas, USA, where different ethnic groups living in the same city shows variation in *H. pylori* genotypes and these genotypes were conserved within each ethnic group (Yamaoka *et al.*, 2000a). Similar study conducted in Kuwait also reported diversity of *H. pylori* among different ethnic groups (Al Qabandi *et al.*, 2005).

4.1.5 Distribution of *H. pylori cagA, babA2, dupA* and *sabA* virulence genes and clinical outcome

H. pylori colonisation is associated with a variety of gastroduodenal pathologies. Although infection is commonly associated with gastritis, the development of clinically significant disease seems to depend on a number of factors, including the virulence of the bacterial strain, the susceptibility of the host and environment cofactors. Several studies have shown that the incidence of gastroduodenal disease related to *H. pylori* vary geographically especially in strain. (Hocker and Hohenberger, 2003; Kusters *et al.*, 2006). In this study, we determined the frequency of *cagA*, *babA2*, *dupA* and *sabA* in dyspeptic patients.

The prevalence of *cagA* is different in every part of the world. The prevalence of *cagA* gene is low in Western countries (Podzorski *et al.*, 2003; Ribeiro *et al.*, 2003) as compared to East Asian countries where the *cagA* are present in more than 90% of cases, irrespective of clinical presentation (Yamaoka *et al.*, 2002). *cagA* has been proposed as a marker for the cag PAI and is linked with more severe clinical outcomes (Yakoob *et al.*, 2013).

The prevalence of *cagA* gene in this study was 69.5%, this is slightly lower than reports from East Asian countries. Studies done locally reported different percentage. Ramelah at al found the prevalence of *cagA* as 94% (Ramelah *et al.*, 2005) while Amjad et al also reported 43%. This divergence within the same country may well be due to differences in sample size, primer sets or the variety of strains within the same country. In addition, the results of this study did not find any association of *cagA* with PUD or gastritis patients, the result of this study is in

agreement with other studies in Asian countries (Zheng *et al.*, 2000; Ramelah *et al.*, 2005) that did not find any association. However, some studies have reported that *cagA* gene was statistically associated with PU (Oliveira *et al.*, 2003; Erzin *et al.*, 2006).

Adherence of *H pylori* to epithelial cells is a significant step in the development of gastroduodenal pathologies. *BabA2* attaches *H. pylori* to these cells, enabling delivery of *VacA* and *cagA* toxins near the gastric epithelium and therefore increasing gastric tissue damage (Gerhard *et al.*, 1999). *H. pylori babA2* positive strains have been reported to be associated with increased risk of gastric cancer and peptic ulcer disease, while *babA2* negative strains have been associated with less severe forms of gastritis (Hocker and Hohenberger, 2003).

The prevalence of the *babA2* genotype in the current study was 41.0%. This result is consistent with a study from China, which found 38.9% of *babA2* in dyspeptic patients (Zheng *et al.*, 2006) but slightly lower than the study from Turkey 53.8% (Erzin *et al.*, 2006). Oliveira *et al*. found that *babA2* was more often found in patients with duodenal ulcer and gastric cancer (Oliveira *et al.*, 2003). In this study there is no gastric cancer case and duodenal ulcer accounts only for 4.8% of this population, this might have contributed to the low prevalence of *babA2* in this study. Gerhad *et al.* have found that *babA2* is associated with peptic ulcer disease in Western populations (Gerhard *et al.*, 1999). The current study did not find any significant association between *babA2* and clinical outcome. This is in agreement with a previous study (Abdollahi *et al.*, 2013) that did not find any link.

In the present study, *dupA*-positive *H. pylori* strains were 24 (22.9%). This data are in line with a study done in Japanese patients 21.3 % (Lu *et al.*, 2005) and slightly lower than a study from China 35.3% (Zhang *et al.*, 2008). The only single study done in Malaysia also found a similar prevalence of 21.3% (Schmidt *et al.*, 2009a). *dupA* has been associated with an increased risk of duodenal ulcer (DU) and a decreased risk for GC (Lu *et al.*, 2005). In contrast to the results of Lu et al (Lu *et al.*, 2005) *dupA* was not associated with duodenal ulcer in this population. These differences in results might be due to strain variation from one region to another. Furthermore DU patients in this study accounts only for 4.8% of the study subject, this limitation impairs a distinct conclusion.

Gastritis patients have a higher distribution of *cagA* (76.7%), *babA2* (74.4%) and *dupA* (75.0%) as compared with PUD. This result is in agreement with a study from Iran, which found higher prevalence of *cagA* gene (73%) in gastritis patients (Dabiri *et al.*, 2009). Others have found higher presence of *cagA* in PUD patients than in NUD (Ramelah *et al.*, 2005; Alaoui Boukhris *et al.*, 2012). This difference might be due to an imbalance in NUD and PUD cases.

H. pylori sialic acid binding adhesin (*sabA*) is widely believed to play a critical role in initial colonization of *H. pylori* and later leading to infection (Mahdavi *et al.*, 2002). *sabA* is essential for attachment and stimulation of human cells via sialyl-Lewis x/a antigens. In a previous study performed on *H. pylori*-infected patients from Colombia and the United States, the prevalence rates of *sabA*-positive isolates were 44% in duodenal ulcer, 66% in gastritis and 70% in gastric cancer (Yamaoka *et al.*, 2006). These study, found that *SabA* positive status was associated with gastric cancer, intestinal metaplasia, and corpus atrophy and negatively

associated with DU (Yamaoka *et al.*, 2006). In a study done in Iran, they found the frequency of *sabA* in both gastritis and duodenal ulcer as 83.3% while the frequency of gastric ulcer and gastric cancer was 86.7% and 100% respectively (Pakbaz *et al.*, 2013).

In the current study, the frequency of *sabA* gene was 43.8%. The frequency of *sabA* in gastritis, GU and DU was 78.3%, 8.7% and 6.5% respectively. However, no significant association was seen between the prevalence of *sabA* genotype and clinical outcomes. Lack of association in this study, might be due to absence of some clinical outcome like gastric cancer, intestinal metaplasia in this patients.

A combinations of the three virulence genes or two were not significantly different among the gastritis, GU, DU and the normal groups in this study, although a study done in Cuba reported a significant association between *cagA* and *babA2* genotypes (Torres *et al.*, 2009).

4.1.6 Distribution of *cagA* EPIYA motif, ethnicity and clinical outcome

The prevalence of *cagA* positive *H. pylori* varies from one geographic region to another. *cagA* is part of a marker for the cag pathogenicity island (PAI) The cag PAI. encodes a type IV secretion system (T4SS) that is responsible for the entrance of *cagA* into host gastric epithelial cells (Ferreira *et al.*, 2014). *CagA* protein can be categorized into two major types, Western and East Asian type, on the basis of the amino acid sequence surrounding the EPIYA motif. EPIYA-A and EPIYA-B are found in both the Western and East Asian *cagA* type protein. The Western *cagA* contain the EPIYA-C motif and the East Asian *cagA* contain the EPIYA-D motif

(Jones *et al.*, 2010). EPIYA motif types are possibly better virulence markers than the *cagA* gene alone to predict clinical outcomes of *H. pylori*-associated pathologies (Hatakeyama and Higashi, 2005).

In the current study, *cagA* subtypes were detected on the basis of significant EPIYA motifs; these subtypes were detected according to (Argent *et al.*, 2005) and (Jones *et al.*, 2009) who used single forward and multiple reverse primers specific to EPIYA-A, EPIYA-B, or consensus sequence of EPIYA-C and EPIYA-D. The current results were confirmed using two reverse primers, namely, *cagA*-West and *cagA*-East, which are specific to EPIYA-C and EPIYA-D, respectively; these primers were designed by (Schmidt *et al.*, 2009b). Sequencing for some samples was also done for confirmation of the result.

In the present study, *cagA* diversity was investigated among clinical isolates from patients of Malay, Chinese, and Indian ethnicity; the observed distribution varied among the patients from these three ethnic groups. EPIYA-A and EPIYA-B were detected in all this current study sample; this result is almost in agreement with that in previously reported studies (Panayotopoulou *et al.*, 2007; Schmidt *et al.*, 2009b). The East Asian strain (EPIYA-ABD) was predominately isolated from Chinese patients (88.9%), whereas the Western strain (EPIYA-ABC) was mainly found in Indians (82.8%) and Malays have mixed strains of both East Asian strain (30.8%) and western strain (53.8%). The current study observed the mixed presence of *H. pylori cagA* types; a statistically significant difference ($P < 0.001$) was observed between ethnicity and *cagA* EPIYA motifs. This result is similar to other studies that show differences of EPIYA motif in different ethnic group. A study conducted by Schmidt et al found that Indian ethnic possessed 93.0% of Western

EPIYA C motifs and majority of Chinese resident in Malaysia and Singapore had East Asian EPIYA ABD type (91.8%). The Malay was found to possess mixed strains. (Schmidt *et al.,* 2009b).

Similar studies conducted in China and India also reflects the same trend where majority of Chinese in China have East Asian ABD type and majority of Indians in India carry Western ABC type (Kumar *et al.*, 2010; Chen *et al.*, 2013). The current study suggests that Chinese and Indian immigrant residents in Malaysia have retained their ethnic background strain.

The East Asian ABD type is more virulent than the Western type and previous studies have reported that *cagA*-ABD type is highly linked to the risk of gastric cancer (Azuma *et al.*, 2004; Jones *et al.*, 2009). Despite the majority of Chinese carrying *cagA*-ABD subtype in the present study, we did not find any cases of gastric cancer or any association with other gastroduodenal diseases (chronic gastritis, GU, or DU). This findings agree with those of (Schmidt *et al.*, 2009b), who did not find any association between the presence of *cagA*-ABD and gastric cancer in Chinese patients.

Southeast Asia was probably free of *H. pylori* infection before major human migrations occurred. Breurec et al. (Breurec *et al.*, 2011a) reported five major types of historical human migration patterns in Southeast Asia: (i) the migration from India introducing hpEurope bacteria to Thailand, Cambodia, and Malaysia; (ii) the migration of the ancestors of Austro-Asiatic speaking people carrying hspEAsia bacteria in Vietnam and Cambodia; (iii) the ancient migration of Thai people from Southern China into Thailand transporting *H. pylori* of the hpAsia2 population; (iv) the migration of Chinese to Thailand and Malaysia in the last 200 years causing the

spread of hspEAsia strain; and (v) the migration of Indians to Malaysia in the last 200 years, introducing hpAsia2 and hpEurope bacteria. These immigration trends probably led to the presence of both Western and East Asian EPIYA motifs in Southeast Asia. This is supported by *H. pylori cagA* EPIYA patterns that show a significant geographic variability and follow patterns of historical human migrations. EPIYA D is a characteristic Asian EPIYA pattern that virtually does not occur in the Western *H. pylori* strains.

The prevalence rates of Western and East Asian *cagA* strains among Malays were 54.79% and 38.36%, respectively indicating presence of mixed strain. This findings differed from those of studies conducted in Japan and China, which reported that East Asian *cagA* is the predominant strain in their population (Azuma *et al.*, 2004; Zhou *et al.*, 2004) but in agreement with other studies in Thailand which found the existence of a mixed strain in their populations (Yamazaki *et al.*, 2005a). However, the origins of the mixed *cagA* strain in ethnic Malays remain unknown; previous studies also suggested that ethnic Malays possibly acquired *H. pylori* from their Indian and Chinese migrant spouses (Graham *et al.*, 2007; Sahara *et al.*, 2012). This theory can also explain the relatively low *H. pylori* infection and other related disease development rates in the Malay population. However, future studies on a larger number of populations need to be performed to confirm this theory.

The PCR amplicons of the EPIYA motifs in the current study ranged in size from 550 to 850bp depending on the number of the C motif repeats present. Although both EPIYA motifs were detected in this study, the number of EPIYA motifs in *H. pylori* from patients with different gastroduodenal diseases was not significantly different. These findings agree with those in other studies, in which

such association was not detected (Schmidt *et al.*, 2009b; Chomvarin *et al.*, 2012). The variety of the EPIYA motifs, especially EPIYA-C, has been linked to the development of gastroduodenal diseases by other researchers (Salih *et al.*, 2010; Shokrzadeh *et al.*, 2010). In the present study, EPIYA-ABCC and EPIYA-ABCCC strains were rare and there were no association with clinical outcomes. The findings is in agreement with other studies that did not find any associations (Basso *et al.*, 2008; Batista *et al.*, 2011).

4.2 Phase II: Genome wide association study

The genetic basis of common human diseases has been the subject of many studies. Genetic factors have been identified in most of the common diseases, but how many more genetic factors are to be found is still an open question. GWAS is a powerful tool that provides a relatively unbiased method of interrogating the genome for susceptibility variants. Unlike candidate gene association studies, the hypothesis-free design of GWAS means that all DNA variants in the genome can be potential targets. Current SNP microarrays can genotype hundreds of thousands of SNPs and other non-polymorphic markers (Robinson, 2010). Numerous common variants are implicated for complex diseases from GWAS, but these only explain a small part of the genetic risk (Cantor *et al.*, 2010).

The common disease-common variant (CDCV) hypothesis postulates that a few common, interacting disease alleles account for most common diseases whereas, the rare variant (CD/RV) hypothesis stated that rare DNA sequence variation at any gene, each with relatively high penetrance, are the major contributors to genetic susceptibility to common diseases. (Hemminki *et al.*, 2008; Schork *et al.*, 2009). In several diseases, GWAS were successful at discovering numerous novel associations in genomic regions that were not suspected to be involved and at demonstrating the role of novel metabolic pathways in these pathologies. However, most of the common variants identified are difficult to link with the disease. Several of the associations concern SNPs that are located in noncoding regions of the genome and even when the signals detected are located in or close to genes, it is usually very difficult to identify the causal variants tagged by the associated SNPs (Saint Pierre and Genin, 2014).

H. pylori colonizes about half of the world population and it is associated with severe gastritis-associated diseases, including peptic ulcer and gastric cancer (Watada *et al.*, 2011). The cause of *H. pylori* infection may be influenced by interactions between the host genetic factors and bacterial virulence factors (Hayashi *et al.*, 2012). Host genetic polymorphisms affecting expression levels of important genes involved in pathogenicity have been demonstrated to influence susceptibility and severity of *H. pylori* infection (McNamara and El-Omar, 2008).

The seroprevalence of *H. pylori* infection varies between and within specific regions and communities of individual countries. Among East Asian countries, the overall seroprevalence rate was 58.07% in China, 39.3% in Japan, 59.6% in South Korea and 54.5% in Taiwan. Among Southeast Asian countries, the reported seroprevalence rate was 35.9% in Malaysia, 31% in Singapore and 57% in Thailand (Fock and Ang, 2010). In Malaysia, three distinct ethnic groups predominate: Malays, Chinese and Indians. The prevalence of *H. pylori* infection varies among the different ethnic groups. There are variations between human populations and, thus, a SNP allele that is common in one geographical or ethnic group may be rare in another group. Genetic variation between ethnicities may influence *H. pylori* susceptibility and the outcome of infection (Schmidt *et al.*, 2011; Maran *et al.*, 2013).

The technology of high-throughput microarrays has developed very dramatically and can identify large number of SNPs in the human genome with low cost. For example, Affymetrix Genome-wide Human Array 6.0 has 906,600 SNPs available for genotyping and can be also used to identify CNVs. This provides the unprecedented opportunity to conduct genome-wide association studies among

unrelated subjects to examine the correlation between SNP allele and a complex disease (Anantharaman and Chew, 2009)

In GWAS, the use of genome-wide significance threshold makes few SNPs surpass the stringent statistical requirement and genetic markers that do not equal or exceed this conservative threshold are generally ignored or neglected unless the biological plausibility is very strong. The threshold level for the discovery of a genetic variant associated with a disease has been put at P-values between 5×10^{-7} – 1×10^{-8} to reduce the probability of a false positive finding (Duggal *et al.*, 2008; Chung *et al.*, 2010). However some studies have used lower threshold.

In order to unravel these modern strategies with high capacity for generating large number of polymorphic sites in the human genome was a necessity. Therefore the current study has made use of the sensitivity and high throughput nature of DNA microarray technology which provides an ideal platform for interrogating up to hundreds of thousands of SNPs in a single array. Present study has used the latest Affymetrix Genome-wide Human Array 6.0 for genotyping.

4.2.1 Genotype- phenotype association analysis of *H. pylori* infection among ethnic groups

GWAS revolutionized the study of human genetics research and the relationship between genotype and phenotype (Leslie *et al.*, 2014). Natural selection acts on phenotypes and indirectly leaves a signal at the molecular level. The connection between the two levels is important because it ties together the effects of

natural selection. Thus, selection for a phenotype can change the genetic variation for specific genes or genomic regions.(O'Connor and Mundy, 2009).

The current genome wide association study has highlighted the genetic factors such as the causative characteristics of SNPs that may lead towards the phenotype changes in the *H. pylori* infected patients. The phenotype characteristics investigated in this study were gastritis and peptic ulcer disease among Malay, Chinese and Indian ethnic group.

This study used Plink (version 1.07) software to do genotype-phenotype association analysis. Plink (version 1.07) is a widespread and computationally efficient software program that offers a comprehensive and well-documented set of automated GWA quality control and analysis tools This software was used as it provides many statistical measures for testing genotype association for case control study. The current study utilizes statistical measures such as minor allele frequency, major allele frequency and P- value that are suited to find genotype-phenotype association.

Manhattan plot is a commonly used method used to visualize GWAS results. Manhattan plot displays a plot of the $-\log_{10}$ (P-value) of the association statistic on the y-axis versus the chromosomal position of the SNP on the x-axis. Because the strongest association have the smallest p –values, their negative logarithms will be the greatest. Therefore, the higher the dots in the Manhattan plot, the stronger the genetic association.

Knowing the most promising SNPs presents a challenge in GWAS study, particularly when hundreds of thousands of association tests were conducted in

finding theses SNPs. Statistical test such as chi-squared (x^2) P value were widely used in GWAS to determine the significant SNPs between case and control (Clarke *et al.*, 2011)

In the current study, the P-value for each SNP was calculated by Plink (version 1.07) software using x^2 test. In statistical significance testing, the P-value is the probability of obtaining a test statistic at least as extreme as the one that was actually observed, assuming that the null hypothesis is true. The null hypothesis is rejected when the P-value is less than the significance level α. When the null hypothesis is rejected, the result is assumed to be significant.

The filtering was also done in order to get high quality genotype data and for enlarging the number of potentially associated SNPs. To achieve this poorly behaving SNPs were effectively eliminated with the SNP filtering parameters; those having minor allele frequency (MAF) > 0.01, those deviating from Hardy-Weinberg equilibrium HWE p-value > 0.001 and SNP call rate > 95%.

4.2.2 Predisposing and protective SNPs in *H. pylori* gastritis phenotype among Indian population

Gastritis refers to inflammation of the gastric mucosa. When a person is infected with *H. pylori* the initial response to infection is the development of an acute gastritis. This acute phase is characterized by the presence of fever, vomiting, nausea and mucosal inflammation, The majority of *H. pylori* infected individuals are asymptomatic despite the on-going gastritis and if *H. pylori* is not eliminated, the acute gastritis progresses to chronic gastritis. Of those infected, a small proportion of

patients infected with *H. pylori* will develop more serious clinical outcomes such as peptic ulcer disease (PUD) and gastric cancer (Kusters *et al.*, 2006).

Among the *H. pylori* infected Indian population, 18 were found to have gastritis and this compared with 18 controls. When association study was conducted in 690, 885 SNPs available for analysis after filtering, few significant SNPs were generated. The most two significant SNP are rs1809578 and rs3770521 with p-value of 9.85×10^{-6} and $1.33 \times^{-5}$ respectively (Figure 3.16 and Table 3.13). rs1809578 ($P=9.85 \times 10^{-6}$) which is located in chromosome 4 and associated with gene BANK1 was the most significant SNP. The minor allele frequency of SNP rs1809578 was 0.38 (38%) and 0.89 (89%) among cases and control respectively (Table 3.13), suggestive that it's protective gene.

BANK1 (B-cell scaffold protein with ankyrin repeats 1) is an adaptor protein that is highly expressed in B-cells. It is involved in the activation of B cells through the B cell receptor signalling cascade. Therefore, an impaired function of this gene would lead to a disease-susceptible state (Zouidi *et al.*, 2014). Polymorphisms in this gene are associated with susceptibility to several autoimmunity diseases. Systemic lupus erythematosus (SLE) is an autoimmune disease and single nucleotide polymorphisms (SNPs) in BANK1 have been reported to be associated with SLE (Kozyrev *et al.*, 2008; Chang *et al.*, 2009).

Single nucleotide polymorphisms (SNPs) in BANK1 have been associated with Rheumatoid arthritis (RA), a chronic systemic autoimmune condition that affects the joints (Orozco *et al.*, 2009). Other study has also found associated SNPs in BANK1 with Systemic sclerosis (SSc) (Rueda *et al.*, 2010).

A relation between rs1809578 in BANK1 and *H. pylori* gastritis among Indian population has not been reported in the past. The current study shows for the first time carriers of the rs1809578 variant allele have a reduced risk of developing *H. pylori* gastritis. Therefore, this gene may play a protective role in *H. pylori* infection among the Indian Population. Further study is necessary to confirm these findings.

Similarly among Indians, rs3770521 ($P=1.33x^{-5)}$ located at chromosome 2 was detected to be significant with *H. pylori* associated gastritis. This SNP has been associated with XRCC5 gene. Double-strand breaks (DSBs) are the most lethal form of DNA damage, and non-homologous end-joining (NHEJ) is the major mechanism for DSB repair (Yang *et al.*, 2011). XRCC5 gene encodes for the protein KU86, an important component of NHEJ pathway and NHEJ deficiencies can lead to increased genomic instability and increased risk for development of Human cancers (Bau *et al.*, 2011; Gorre *et al.*, 2014).

A human cell is injured by hundreds of thousands exogenous and endogenous DNA damage per day. This is maintained mainly by DNA repair pathways which can sense all kinds of DNA damage and repair them. If the cell does not repair DSB well, it leads to genomic instability. Therefore, XRCC5 play important role in both genomic stability and human carcinogenesis (Yang *et al.*, 2011). Several studies have reported association between some of the SNPs of XRCC5 and the susceptibility to different type of cancers (Bau *et al.*, 2011)

A study conducted in Taiwan, where the highest incidence of colorectal cancer is found, reported that the XRCC5 rs828907 polymorphism was associated

with increased colorectal cancer, while the XRCC5 rs11685387 and rs9288518 genotypes have no similar association (Yang *et al.*, 2009). A study done in oral cancer patients also found that XRCC5 rs828907 was associated with oral cancer susceptibility, However, they did not find any association with rs11685387 or rs9288518 (Hsu *et al.*, 2009). Dong and colleagues have also found association between *XRCC5* gene and those subjects with familial history of gastric cancer (Bau *et al.*, 2011).

H. pylori as a major cause of gastritis and gastritis could progress to gastric cancer. *H. pylori* babA-mediated binding to Leb contributes to formation of double stranded DNA breaks in host cell lines and may promote cancer-associated gene mutations (Testerman and Morris, 2014). Therefore, SNP rs3770521 of XRCC5 gene might play a role in causing occurrence of *H. pylori* gastritis among Indian population. The allele frequency in cases was 47% (Table 3.13)

The current study also detected SNPs among 2 cases of peptic ulcer among Indian population. However, none of these SNPs are statistically significant (P >0.05) (Table 3.14). This finding might be due to low cases of this phenotype in this study.

As per our knowledge there was no association study conducted on BANK1 and XRCC5 gene and its link with *H. pylori* infection. Therefore, the current study highlights novel findings and opens a new avenue for more studies in this field.

4.2.3 Predisposing and protective SNPs in *H. pylori* gastritis phenotype among Malay population

The process that mediates *H. pylori*-induced damage is gastritis with its associated humoral and cell-mediated immune mechanisms. The degree and distribution of this gastritis eventually determine the clinical outcome (McNamara and El-Omar, 2008). The only GWAS study done among Kelantanese Malay using Affymetrix 50k found that ethnic Malays are genetically susceptible to *H. pylori* infection and maybe mediated through a genetic variation in the DCC gene (Maran *et al.*, 2013).

In the current study, a total of (9/38) *H. pylori* infected patients were identified with *H. pylori* gastritis among Malay population. Association study was done among 9 cases and 8 controls. The most significant SNP was rs7042986 (P=0.0001) and located in chromosome 9 and associated with gene SMARCA2 (Table 3.15 and Figure 3.18).

SMARCA2 gene encode encodes BRM, which are responsible for the DNA dependent ATPase activity of the SWI/SNF complex. This activity is essential to regulate the accessibility of transcription factors to their DNA targets for expression and therefore important in cancer development (Amankwah *et al.*, 2013). SNPs in SMARCA2 gene have been linked with schizophrenia and Human cancer development. Polymorphisms in SMARCA2 gene have been linked with Schizophrenia (Loe-Mie *et al.*, 2010) and human gastric cancer (Yamamichi *et al.*, 2007).

The identification of SNP rs7042986 associated with gene SMARCA2 among *H. pylori* gastritis in Malay population suggest that this SNP may play a role in *H. pylori* infection. Furthermore the allele frequency among cases was 89% (Table 3.15).

On the other hand, the current study also identified SNP rs3776349 (P=0.0001) associated with gene ARHGAP26 among *H. pylori* gastritis in Malay population (Table 3.15 and Figure 3.18). ARHGAP26 codes for a protein acting in particular as a negative regulator of Rho A, a member of Rho family, which cooperates in Rho kinase pathways that reduce the endothelial integrity. Polymorphisms in ARHGAP26 have been associated with malaria infection (Milet *et al.*, 2010).

SNP rs3776349 detected in this population seems to be protective against *H. pylori* infection. Minor allele frequency among cases is only 0.16 (16%) (Table 3.15). This suggests that rs3776349 located among the cases does not play role towards the gene. Therefore, further suggesting rs3776349 detected among patients with *H. pylori* gastritis might play a protective role in *H. pylori* infection.

The current study for the first time show link between SMARCA2 and ARHGAP26 and *H. pylori* infection. Therefore, these unique findings call for more research to validate this work.

4.2.4 Predisposing SNPs in *H. pylori* gastritis phenotype among Chinese population

H. pylori infection results in various clinical conditions ranging from chronic gastritis and ulceration to gastric adenocarcinoma. Among the multi-ethnic population of Malaysia, Indians have a higher *H. pylori* prevalence as compared with Chinese and Malays. In spite of the high prevalence of *H. pylori*, Indians have a relatively low incidence of peptic ulcer disease and gastric cancer. In contrast, gastric cancer and peptic ulcer disease incidence is high in Chinese (Schmidt *et al.*, 2011; Gunaletchumy *et al.*, 2014).

In the current study, there were (9/38) *H. pylori* gastritis cases identified in Chinese population. SNPs analysis was conducted among 9 cases and 8 controls. The most significant SNP detected was rs10860808 with p-value of 0.0002 (Figure 3.19 and Table 3.16). SNP rs10860808 is located in chromosome 12 and this SNP is associated with DRAM1 gene.

DRAM1 (DNA damage-regulated autophagy modulator 1) is a TP53 target gene that modulates autophagy and apoptosis. Autophagy and apoptosis have important roles in many biological functions of cells and are involved in pathogenesis of many diseases (Guan *et al.*, 2015). The mechanism by which DRAM1 promotes autophagy is not clear. It is proposed that DRAM1 may exert its effects on autophagy through lysosomes, given the fact as a lysosomal membrane protein (Zhang *et al.*, 2013).

A genome-wide association study conducted on Systemic lupus erythematosus (SLE) in Chinese Han populations reported genetic variants in

DRAM1 as associated with the SLE disease (Yang *et al.*, 2013). Similarly, a study conducted by Laforge revealed that DRAM controls lysosomal membrane permeabilization (LMP); the depletion of p53 and DRAM indeed prevents LMP and cell death in HIV-infected CD4 + T cells (Laforge *et al.*, 2013).

The current study also detected SNPs among 2 cases of peptic ulcer among Chinese population. However, none of these SNPs are statistically significant ($P > 0.05$) (Table 3.17). This finding might be attributed to low cases of this phenotype in this study.

Considering the above facts, the current study identified SNP rs10860808 as susceptible gene among the Chinese Population infected with *H. pylori* gastritis. The allele frequency among the control was 0.86 (86%) (Table 3.16). As per our knowledge there was no previous study conducted on DRAM1 gene and its susceptibility with *H. pylori* infection. Therefore, the current study highlights novel findings.

4.2.5 Novelty and limitations of the study

The novelty of this study is the utilization of a relatively new technology, the whole genome SNP analysis which allows the study of an individual whole genome variant and associate it to disease. GWAS have emerged as a popular tool for identifying genetic variants that are associated with disease risk. This study involves large number of SNPs, on the order of hundreds of thousands, in individuals with the disease (cases) and healthy controls with the goal of identifying individual loci that are associated with the outcome. The greatest strength of this study was using the

Affymetrix Genome-wide Human Array 6.0 which can genotype 906, 600 SNPs. This study identified new novel susceptible and protective SNPs among three ethnic group (Malay, Indian and Chinese) infected with *H. pylori* gastritis.

The strength of this study is that also, it was able to determine the distribution of *H. pylori cagA*, *dupA*, *babA*2, *SabA* and *cagA* EPIYA motifs in *H. pylori* infected patients by using PCR; additionally the study identified the distribution of these genes among different ethnic group in Malaysia and determined their association with clinical outcome. This study also shows a significant geographic variability of *H. pylori cagA* EPIYA and indicates patterns of human migrations. EPIYA D is a characteristic Asian EPIYA pattern that virtually does not occur in the Western *H. pylori* strains.

The main limitation of this study was the small sample size and strict inclusion criteria of selecting three generation from each group. There was also lack of cooperation from the participants in giving all needed samples. Therefore, it will be highly important to replicate the findings of this study in a larger population.

Chapter 5: Conclusion

5.1 Conclusion

This study has identified SNPs rs3770521 of XRCC5 gene, rs7042986 of SMARCA2 and rs10860808 of DRAM1 gene as the susceptible SNPs to *H. pylori* infection among gastritis patients of Indian, Malay and Chinese ethnicity respectively. This study has also identified two protective SNPs rs1809578 of gene BANK1 and rs3776349 of gene ARHGAP26 among *H. pylori* gastritis patients of Indian and Malay ethnicity respectively. Identification of the above new SNPs among different ethnic groups highlights the novelty of the current study. The current study also determined *H. pylori cagA, dupA, babA2 SabA* and cagA EPIYA motifs distribution of bacterial genes. Moreover it showed the predominant cagA EPIYA motif varied significantly between the three ethnic groups, with Indians representing a Western strain (EPIYA A-B-C), Chinese representing a typically East Asian strain (EPIYA A-B-D) and malays having mixed strain. This support the contention that ethnic Malays were an *H. pylori*-free population and acquired *H. pylori* from other populations. There was statistically significant difference between race and *cagA* EPIYA motifs. Another finding of this study was that the new Atlas *H. pylori* antigen test showed high sensitivity and specificity and can be used as an alternative method in the diagnosis of *H. pylori* infection in adults.

5.2 Future recommendations

The above findings have opened a number of avenues for future studies.

The absence of a significant correlation between the virulence genes analysed and the development of gastroduodenal disease might be due to the small number of patients with each disease condition in this study. It may be that a large population of patients must be studied to reveal statistically significant relations between *H. pylori* virulence genes and patterns of clinical disease. The current study also lack GC cases that are associated with *H. pylori* infection, future study with this case might reveal more important result.

H. pylori colonize about half of the world population and currently few studies on GWAS were conducted, therefore, there is a need for more research on this bacterium. Although the current study identified new SNPs associated with *H. pylori*, the number of sample examined was small and thus further studies with larger numbers of patients are required to verify this finding.

GWAS have become the primary approach for identifying genes with common variants influencing complex diseases. Despite considerable progress, the common variations identified by GWAS account for only a small fraction of disease heritability and are unlikely to explain the majority of phenotypic variations of common diseases. A potential source of the missing heritability is the contribution of rare variants. Next-generation sequencing technologies will be an alternative as it will detect millions of novel rare variants.

Ethnicity plays a role in the disease's progress on genetic variation in the susceptibility or resistance, therefore, a separate study on GWAS that focus on each ethnic group is highly recommended.

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APPENDICES

Appendix A: SNP result

Appendix B: List of published manuscripts

Appendix C: List of Poster and oral Presentation

Appendix D: Study approval

Appendix E: Data collection form (Data sheet questionnaire Malay& English))

Appendix F: Patients Information consent form (English A and Malay B Languages)

APPENDIX A1: SNP RESULT- INDIAN GASTRITIS

CHR	SNP	BP	A1	F_A	F_U	P
4	rs1809578	1.03E+08	T	0.3824	0.8889	9.85E-06
2	rs3770521	2.17E+08	A	0.4722	0.02778	1.33E-05
5	rs6523782	43258528	A	0.1111	0.5833	2.57E-05
2	rs10182201	2.17E+08	G	0.4444	0.02778	3.15E-05
5	rs557302	78377334	G	0.6667	0.1944	5.21E-05
11	rs2846724	1.03E+08	C	0.6667	0.1944	5.21E-05
19	rs741587	34167905	A	0.05556	0.4722	6.05E-05
4	rs17060468	1.75E+08	A	0.4444	0.8889	6.33E-05
8	rs4921941	18459588	G	0.5	0.08333	0.000101
4	rs6822974	63006443	G	0.3333	0.7941	0.000105
7	rs2522217	1.12E+08	T	0.8333	0.3889	0.00011
13	rs7995700	75644644	G	0.6111	0.1667	0.00011
14	rs41443946	82044951	C	0.8333	0.3889	0.00011
3	rs1597314	1.66E+08	G	0.6875	0.2222	0.000115
22	rs9616423	49486691	G	0.1	0.5625	0.00012
1	rs783622	42366988	G	0.6389	0.1944	0.000131
1	rs2147904	42371414	C	0.6389	0.1944	0.000131
1	rs416190	1.15E+08	G	0.6389	0.1944	0.000131
5	rs404220	35395245	T	0.8056	0.3611	0.000131
12	rs4768143	47583343	C	0.6389	0.1944	0.000131
12	rs10881070	47584050	T	0.6389	0.1944	0.000131
12	rs10785668	47584431	G	0.6389	0.1944	0.000131
14	rs10151039	82035725	A	0.8056	0.3611	0.000131
18	rs206473	10295509	T	0.3611	0.8056	0.000131
18	rs12607576	13246168	T	0.1944	0.6389	0.000131
18	rs206474	10295388	A	0.6389	0.1944	0.000131
18	rs9304221	38002661	A	0.8056	0.3611	0.000131
18	rs12327278	38011902	T	0.8056	0.3611	0.000131
19	rs8110889	34177770	C	0.05556	0.4444	0.000139
11	rs7129777	1.03E+08	C	0.3333	0.7778	0.000148
11	rs7129790	1.03E+08	G	0.3333	0.7778	0.000148

12	rs2024303	8273765	A	0.3333	0.7778	0.000148
12	rs2110067	8268997	C	0.6667	0.2222	0.000148
1	rs7543621	1.68E+08	T	0.4722	0.8889	0.00015
23	rs5976268	1.38E+08	C	0.8571	0.3158	0.00015
4	rs7667876	32953000	C	0.9333	0.5	0.000153
1	rs1495845	2.28E+08	C	0.25	0.6944	0.000159
1	rs10495276	2.28E+08	T	0.25	0.6944	0.000159
1	rs7528094	2.28E+08	A	0.75	0.3056	0.000159
1	rs3010186	2.28E+08	G	0.75	0.3056	0.000159
2	rs1477450	1.18E+08	A	0.75	0.3056	0.000159
4	rs4446347	63060580	C	0.75	0.3056	0.000159
1	rs3104202	2.28E+08	G	0.2333	0.7059	0.00016
6	rs9328228	4253351	T	0.9722	0.6111	0.000162
19	rs11672374	36670298	G	0.02778	0.3889	0.000162
7	rs422011	1.08E+08	C	0.2778	0.7222	0.000162
9	rs7856591	94281499	T	0.5	0.08824	0.000172
1	rs3924486	25892880	T	0.5333	0.1111	0.000202
23	rs5959306	78592663	C	0.8966	0.4091	0.000203
4	rs4385063	1.03E+08	G	0.4444	0.8611	0.000205
4	rs4337734	1.03E+08	A	0.4444	0.8611	0.000205
5	rs27052	1470069	G	0.5556	0.1389	0.000205
5	rs27053	1470578	G	0.5556	0.1389	0.000205
22	rs134897	42681860	T	0.1389	0.5556	0.000205
22	rs134898	42682557	T	0.1389	0.5556	0.000205
22	rs112603	42688085	A	0.1389	0.5556	0.000205
1	rs1931077	56870735	C	0.3824	0.02778	0.00021
8	rs767763	13200021	G	0.4375	0.05556	0.000211
4	rs2631271	1.03E+08	G	0.4412	0.875	0.000219
12	rs11105501	90482636	T	0.7188	0.2647	0.000225
5	rs10070532	88764215	A	0.08333	0.4722	0.00023
6	rs1112062	1.48E+08	A	0.9167	0.5278	0.00023
7	rs2665452	97447083	G	0.08333	0.4722	0.00023
7	rs1912445	97447728	T	0.08333	0.4722	0.00023

7	rs2530132	97448726	G	0.08333	0.4722	0.00023
12	rs12319957	98785226	A	0.9167	0.5278	0.00023
17	rs1471616	33575120	A	0.08333	0.4722	0.00023
18	rs616643	8836488	A	0.4722	0.08333	0.00023
20	rs293727	31933064	C	0.9706	0.6111	0.000249
1	rs3748806	2.28E+08	C	0.4167	0.8333	0.000261
3	rs1868510	54165949	A	0.5833	0.1667	0.000261
7	rs6966276	36936043	C	0.8333	0.4167	0.000261
13	rs9318312	75591505	T	0.4167	0.8333	0.000261
13	rs1340900	75617619	G	0.4167	0.8333	0.000261
13	rs1340902	75626490	A	0.4167	0.8333	0.000261
13	rs10454657	75591823	T	0.5833	0.1667	0.000261
13	rs9600419	75599117	G	0.5833	0.1667	0.000261
13	rs1417549	75601353	T	0.5833	0.1667	0.000261
13	rs4885263	75601424	G	0.5833	0.1667	0.000261
13	rs7984770	75624170	T	0.5833	0.1667	0.000261
13	rs1536284	75625815	T	0.5833	0.1667	0.000261
15	rs2869030	78711803	G	0.1667	0.5833	0.000261
4	rs7441657	32952455	G	0.8824	0.4722	0.000261
7	rs3819740	1.08E+08	A	0.8824	0.4722	0.000261
22	rs2058554	21002437	T	0.5882	0.1667	0.000264
7	rs12704482	89390440	A	0.9167	0.5294	0.000271
3	rs17072275	65073645	A	0.375	0.02778	0.000279
8	rs2513929	1.04E+08	T	0.5294	0.1176	0.000285
11	rs12277302	14125753	C	0.5294	0.1176	0.000285
1	rs2786487	42368339	C	0.625	0.1944	0.000293
9	rs7029743	1.11E+08	C	0.6875	0.25	0.0003
5	rs6896861	17727683	A	0.3438	0.7778	0.000304
1	rs2506992	48097805	C	0.4167	0.05556	0.000309
3	rs807197	53615698	C	0.05556	0.4167	0.000309
4	rs16851141	75260332	C	0.4167	0.05556	0.000309
7	rs6980274	89377807	C	0.9444	0.5833	0.000309
7	rs7805912	1.35E+08	A	0.4167	0.05556	0.000309

7	rs11980027	1.35E+08	C	0.4167	0.05556	0.000309
10	rs11202117	88431526	G	0.4167	0.05556	0.000309
10	rs11202121	88432328	G	0.4167	0.05556	0.000309

APPENDIX A1I: SNP RESULT- MALAY GASTRITIS

CHR	SNP	BP	A1	F_A	F_U	P
9	rs7042986	2184258	T	0.8889	0.25	0.000158
5	rs3776349	142430200	G	0.1667	0.8125	0.000166
16	rs1965229	57721146	G	0.1667	0.8125	0.000166
12	rs2706309	60127590	T	0.0625	0.6875	0.000261
9	rs2498430	71684702	G	0.6875	0.0625	0.000261
2	rs7571732	223538316	C	0.3333	0.9375	0.000297
7	rs854569	94950055	G	0.3333	0.9375	0.000297
23	rs5934677	6868042	T	0.1667	0.9091	0.00037
3	rs1603663	22036489	G	0.05556	0.625	0.000396
5	rs295669	58147456	C	0.05556	0.625	0.000396
10	rs12258845	6915075	A	0.05556	0.625	0.000396
10	rs10508317	6916628	A	0.05556	0.625	0.000396
12	rs10902528	132103518	C	0.05556	0.625	0.000396
14	rs6573468	63453139	A	0.05556	0.625	0.000396
14	rs1950975	63453773	G	0.05556	0.625	0.000396
16	rs13333767	80666221	T	0.05556	0.625	0.000396
4	rs11726313	183579487	T	0.1875	0.8125	0.000407
5	rs10064840	81246707	T	0.8125	0.1875	0.000407
2	rs16987297	20219369	G	0.2778	0.875	0.000464
2	rs13015993	217625523	A	0.2778	0.875	0.000464
2	rs10170846	223517705	C	0.2778	0.875	0.000464
2	rs3754907	20251758	C	0.7222	0.125	0.000464
5	rs1862175	121532861	T	0.2778	0.875	0.000464
5	rs2190779	142437930	G	0.2778	0.875	0.000464
6	rs2490445	12544672	C	0.2778	0.875	0.000464
6	rs438258	16405710	A	0.2778	0.875	0.000464
14	rs10138062	103218818	G	0.7222	0.125	0.000464

16	rs4889409	81866586	C	0.7222	0.125	0.000464
18	rs2848745	39057725	G	0.7222	0.125	0.000464
1	rs1418444	202978032	C	0.1111	0.6875	0.000557
1	rs1774847	202994268	T	0.1111	0.6875	0.000557
2	rs840950	65718815	C	0.1111	0.6875	0.000557
3	rs924663	59411413	G	0.1111	0.6875	0.000557
4	rs2728124	89006160	A	0.1111	0.6875	0.000557
5	rs11955771	1720781	C	0.1111	0.6875	0.000557
5	rs2354125	1720938	G	0.8889	0.3125	0.000557
5	rs6866823	52186571	G	0.8889	0.3125	0.000557
9	rs10978962	110412647	T	0.1111	0.6875	0.000557
9	rs10122778	2180603	C	0.8889	0.3125	0.000557
9	rs10738600	2186460	C	0.8889	0.3125	0.000557
10	rs1327249	6651563	A	0.1111	0.6875	0.000557
10	rs10830179	129594596	A	0.1111	0.6875	0.000557
12	rs1795884	60119493	A	0.1111	0.6875	0.000557
12	rs2706306	60129896	T	0.1111	0.6875	0.000557
12	rs10877338	60176579	C	0.1111	0.6875	0.000557
12	rs10877342	60204776	C	0.1111	0.6875	0.000557
15	rs1869133	58877599	G	0.1111	0.6875	0.000557
16	rs12446683	48729383	C	0.1111	0.6875	0.000557
16	rs1508212	65279386	G	0.1111	0.6875	0.000557
16	rs8061623	81862574	C	0.1111	0.6875	0.000557
16	rs11150416	81863593	A	0.1111	0.6875	0.000557
16	rs11077189	7535158	T	0.8889	0.3125	0.000557
16	rs11077191	7535456	G	0.8889	0.3125	0.000557
16	rs4787033	7539452	C	0.8889	0.3125	0.000557
7	rs292706	134988792	C	0.6875	0.07143	0.00059
2	rs823539	2486192	G	0.2222	0.8125	0.000591
2	rs3754908	20252270	G	0.2222	0.8125	0.000591
2	rs6739960	30889292	C	0.2222	0.8125	0.000591
2	rs7565899	30902999	C	0.2222	0.8125	0.000591
2	rs16863911	223443761	C	0.2222	0.8125	0.000591

3	rs6771793	103098838	T	0.2222	0.8125	0.000591
6	rs164534	94057609	C	0.7778	0.1875	0.000591
7	rs508733	78456168	G	0.7778	0.1875	0.000591
8	rs4350094	143378869	T	0.2222	0.8125	0.000591
9	rs6477714	112367400	T	0.7778	0.1875	0.000591
9	rs4490914	112386097	G	0.7778	0.1875	0.000591
10	rs12247441	33851964	C	0.2222	0.8125	0.000591
11	rs875981	130903093	T	0.2222	0.8125	0.000591
12	rs2712542	127730787	C	0.2222	0.8125	0.000591
14	rs7160202	103560586	C	0.7778	0.1875	0.000591
15	rs12594951	42934631	A	0.7778	0.1875	0.000591
23	rs6639786	7161475	T	0.09091	0.8182	0.000614
23	rs4595309	7180530	G	0.1667	0.9	0.000614
1	rs1184068	39279324	G	0.1667	0.75	0.000628
2	rs3770371	80851681	C	0.1667	0.75	0.000628
3	rs5970564	104183552	C	0.1667	0.75	0.000628
3	rs4686710	185633136	C	0.1667	0.75	0.000628
5	rs6873602	174539068	T	0.1667	0.75	0.000628
5	rs12109464	174524388	A	0.8333	0.25	0.000628
5	rs12516874	174525244	T	0.8333	0.25	0.000628
6	rs9476843	9782396	A	0.1667	0.75	0.000628
6	rs2758455	114221955	G	0.1667	0.75	0.000628
6	rs1883181	9776984	C	0.8333	0.25	0.000628
8	rs354519	15526849	T	0.1667	0.75	0.000628
8	rs12549961	84393167	C	0.1667	0.75	0.000628
8	rs11988550	143389521	G	0.1667	0.75	0.000628
8	rs11167137	143394208	G	0.1667	0.75	0.000628
11	rs1943733	84600597	A	0.1667	0.75	0.000628
13	rs11069498	103503322	A	0.1667	0.75	0.000628
13	rs9559849	111243736	A	0.1667	0.75	0.000628
14	rs7155575	103582802	T	0.1667	0.75	0.000628
15	rs8031642	22799908	T	0.1667	0.75	0.000628
15	rs4775730	48300178	T	0.1667	0.75	0.000628

16	rs876973	65283792	G	0.1667	0.75	0.000628
11	rs10790283	118975927	G	0.3333	0.9286	0.000671
19	rs9749349	1329639	G	0.3333	0.9286	0.000671
1	rs6668167	242766826	T	0.6429	0.0625	0.000768
12	rs7296507	121847135	G	0.3571	0.9375	0.000768
2	rs13006628	52892471	G	0.0625	0.625	0.000809
5	rs2644676	174934623	C	0.375	0.9375	0.000809

APPENDIX AII1: SNP RESULT- CHINESE GASTRITIS

CHR	SNP	BP	A1	F_A	F_U	P
12	rs10860808	1.02E+08	A	0.8571	0.1875	0.000253
19	rs1216115	22709636	A	0.1429	0.8125	0.000253
3	rs7611730	19449092	C	0.2143	0.875	0.000269
5	rs3844310	34819280	C	0.2143	0.875	0.000269
5	rs7722035	1.42E+08	C	0.2143	0.875	0.000269
17	rs356054	6443244	G	0.2143	0.875	0.000269
17	rs356053	6444676	T	0.2143	0.875	0.000269
18	rs3893060	44309781	T	0.2143	0.875	0.000269
3	rs10935257	1.37E+08	G	0.1	0.8125	0.000392
9	rs2900570	1.17E+08	G	0.08333	0.75	0.000465
1	rs11166274	1E+08	A	0.2857	0.9286	0.000497
5	rs37427	34934323	T	0.07143	0.6875	0.00059
5	rs37437	34954588	T	0.07143	0.6875	0.00059
5	rs163723	34956294	C	0.07143	0.6875	0.00059
5	rs10051972	75244914	A	0.07143	0.6875	0.00059
5	rs7719354	1.07E+08	C	0.07143	0.6875	0.00059
5	rs2116822	1.07E+08	G	0.07143	0.6875	0.00059
6	rs7741604	20731524	C	0.07143	0.6875	0.00059
6	rs6940223	1.49E+08	T	0.07143	0.6875	0.00059
11	rs1792378	1.15E+08	A	0.07143	0.6875	0.00059
11	rs1712790	1.15E+08	T	0.07143	0.6875	0.00059
11	rs7483725	1.15E+08	A	0.07143	0.6875	0.00059

11	rs1792379	1.15E+08	G	0.07143	0.6875	0.00059
11	rs4145058	1.15E+08	A	0.07143	0.6875	0.00059
20	rs4811928	56412456	T	0.07143	0.6875	0.00059
20	rs6099795	56414264	C	0.07143	0.6875	0.00059
20	rs7273716	56426201	G	0.07143	0.6875	0.00059
20	rs2225318	56436209	G	0.07143	0.6875	0.00059
5	rs6895634	34807928	C	0.2143	0.8571	0.000649
11	rs10891731	1.15E+08	C	0.1429	0.7857	0.000649
12	rs10748401	45152546	C	0.2143	0.8571	0.000649
16	rs171634	3478866	A	0.1429	0.7857	0.000649
9	rs10970191	31183097	C	0.3333	0.9375	0.000705
16	rs1555001	3117354	T	0.3333	0.9375	0.000705
1	rs10925571	2.38E+08	C	0.3571	0.9375	0.000768
1	rs12070012	2.38E+08	C	0.3571	0.9375	0.000768
1	rs4659831	2.38E+08	G	0.3571	0.9375	0.000768
1	rs12058299	2.38E+08	T	0.3571	0.9375	0.000768
1	rs12058346	2.38E+08	T	0.3571	0.9375	0.000768
2	rs11683661	1.25E+08	C	0.3571	0.9375	0.000768
2	rs7589170	2.28E+08	C	0.3571	0.9375	0.000768
2	rs6545008	48196989	C	0.6429	0.0625	0.000768
2	rs11682596	1.25E+08	A	0.6429	0.0625	0.000768
2	rs17011466	1.25E+08	T	0.6429	0.0625	0.000768
4	rs16890905	9916662	T	0.3571	0.9375	0.000768
4	rs978335	1.34E+08	C	0.6429	0.0625	0.000768
5	rs2962615	2689899	C	0.3571	0.9375	0.000768
6	rs9328053	1444295	T	0.3571	0.9375	0.000768
6	rs1922938	1446047	A	0.3571	0.9375	0.000768
7	rs17360344	24160169	T	0.6429	0.0625	0.000768
7	rs177967	31256123	A	0.6429	0.0625	0.000768
7	rs960434	31292900	G	0.6429	0.0625	0.000768
9	rs10738866	31183385	G	0.3571	0.9375	0.000768
9	rs7028093	31205795	A	0.3571	0.9375	0.000768
9	rs10759662	1.17E+08	A	0.3571	0.9375	0.000768

9	rs12351269	16806521	C	0.6429	0.0625	0.000768
9	rs2151521	31185279	C	0.6429	0.0625	0.000768
9	rs1157981	31191703	C	0.6429	0.0625	0.000768
9	rs9298930	31193235	A	0.6429	0.0625	0.000768
9	rs10813508	31196218	G	0.6429	0.0625	0.000768
9	rs7869638	31196675	G	0.6429	0.0625	0.000768
9	rs10758036	31208851	G	0.6429	0.0625	0.000768
9	rs10758037	31208933	G	0.6429	0.0625	0.000768
9	rs10970218	31224296	G	0.6429	0.0625	0.000768
9	rs1930926	31232704	T	0.6429	0.0625	0.000768
9	rs10982004	1.17E+08	C	0.6429	0.0625	0.000768
10	rs16920386	66551141	T	0.3571	0.9375	0.000768
10	rs10999746	73007924	A	0.3571	0.9375	0.000768
10	rs7899034	73010064	G	0.3571	0.9375	0.000768
10	rs12266036	73011159	G	0.3571	0.9375	0.000768
10	rs10823717	73046177	G	0.3571	0.9375	0.000768
10	rs11194744	1.11E+08	T	0.6429	0.0625	0.000768
10	rs4429002	1.11E+08	A	0.6429	0.0625	0.000768
10	rs10884819	1.11E+08	A	0.6429	0.0625	0.000768
10	rs7904693	1.11E+08	T	0.6429	0.0625	0.000768
11	rs1894160	20566453	C	0.3571	0.9375	0.000768
11	rs10833356	20567791	T	0.3571	0.9375	0.000768
11	rs10834906	26115566	C	0.6429	0.0625	0.000768
12	rs1863961	20335201	T	0.3571	0.9375	0.000768
12	rs12367911	30316002	G	0.6429	0.0625	0.000768
12	rs10773661	1.3E+08	G	0.6429	0.0625	0.000768
13	rs4411366	27303847	A	0.3571	0.9375	0.000768
13	rs2149134	27319063	C	0.3571	0.9375	0.000768
14	rs1953851	45089605	T	0.3571	0.9375	0.000768
14	rs1431055	34584081	G	0.6429	0.0625	0.000768
17	rs917443	33004089	T	0.3571	0.9375	0.000768
18	rs7231836	11183982	C	0.6429	0.0625	0.000768
19	rs1670725	50773134	C	0.3571	0.9375	0.000768

19	rs1875421	51798572	A	0.3571	0.9375	0.000768
19	rs1399834	51797326	G	0.6429	0.0625	0.000768
20	rs6045843	19098511	T	0.3571	0.9375	0.000768
22	rs738418	50082971	G	0.3571	0.9375	0.000768
22	rs9616340	50086808	A	0.3571	0.9375	0.000768
3	rs12494488	1.89E+08	A	0.25	0.875	0.000805
11	rs11033999	37189731	C	0.25	0.875	0.000805
20	rs2295573	18609314	C	0.5714	0	0.000818
1	rs9426783	22249057	C	0.1429	0.75	0.000883
1	rs10158567	2.41E+08	A	0.1429	0.75	0.000883
1	rs4131804	2.41E+08	T	0.1429	0.75	0.000883
2	rs4849907	1.21E+08	T	0.1429	0.75	0.000883

APPENDIX B: LIST OF PUBLISHED MANUSCRIPTS

PAPER I

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Diagnostic value of Atlas Helicobacter Pylori stool Antigen Test

RESEARCH ARTICLE

Evaluation of the Atlas *Helicobacter pylori* Stool Antigen Test for Diagnosis of Infection in Adult Patients

Hussein Ali Osman[1], Habsah Hasan[1], Rapeah Suppian[2], Norhaniza Bahar[3], Nurzam Suhaila Che Hussin[4], Amry Abdul Rahim[5], Syed Hassan[6], Dzulkarnaen Zakaria Andee[6], Bin-Alwi Zilfalil[7]*

Abstract

Background: *Helicobacter pylori* (*H.pylori*) is one of the most important causes of dyspepsia and gastric cancer and diagnosis can be made by invasive or non-invasive methods. The Atlas *Helicobacter pylori* antigen test is a new rapid non-invasive method which is simple to conduct. The aim of this study was to determine its sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy. Materials and Methods: This prospective study was conducted between July 2012 and December 2013. Stool samples of 59 dyspeptic patients who underwent upper endoscopy were evaluated for *H. pylori* stool antigen. Results: From the 59 patients who participated in this study, there were 36 (61%) males and 23 (39%) females. *H. pylori* was diagnosed in 24 (40.7%) gastric biopsies, 22 (91.7 %) of these being positive for the Atlas *H. pylori* antigen test. The sensitivity, specificity, PPV, NPV and accuracy were 91.7%, 100%, 100%, 94.6% and 96.6% respectively. Conclusions: The Atlas *H. pylori* antigen test is a new non-invasive method which is simple to perform and avails reliable results in a few minutes. Thus it can be the best option for the diagnosis of *H. pylori* infection due to its high sensitivity and specificity.

Keywords: *Helicobacter pylori*- sensitivity - specificity - Atlas *H. pylori* antigen test

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Introduction

Helicobacter pylori(*H. pylori*) is a Gram-negative microaerophilic bacterium and one of the most common bacterial pathogens of humans that infects more than half of the world's population (Amjad et al., 2010; Zhang et al., 2014). The bacteria has worldwide distribution and the prevalence ranges from 25% in developed countries to more than 90% in developing areas, but not all infected individuals eventually developed the disease (Miernyk et al., 2011; Ghotaslou et al., 2013).

The prevalence of *H. pylori* infection varies widely by geographic area, age, race, and socioeconomic status (Brown et al., 2002). *H. pylori* infection is associated with chronic gastritis, gastric or duodenal ulcer, gastric cancer and MALT-lymphoma (Ben Mansour et al., 2010; Zhao et al., 2012). *H. pylori* was classified as a class I carcinogen in humans by a working group of the World Health Organization International Agency for Research on Cancer (IARC) based on various epidemiological studies (Khalilpour et al., 2013)

There seems to be no firm agreement as to which method should be used as gold standard for the detection of *H. pylori* infection (Redeen et al., 2011). Gastric biopsy based tests which include culture, histology and the rapid urease test (RUT) are considered the standard diagnostic tests (Al-Humayed et al., 2008; Kalem et al., 2010). However, these tests necessitate an upper gastrointestinal endoscopy and are considered invasive tests.

Non-invasive tests include the urea breath tests (UBT) and serology and stool antigen test (Bhewa et al., 2007; Redeen et al., 2011). Urea breath tests and stool antigen test can detect active infection while serology test does not differentiate between active infection and exposure to *H. pylori* (Ricci et al., 2007; Peng et al., 2009).

The choice of a given testing strategy is influenced by sensitivity, specificity, the clinical circumstances and the cost-effectiveness of the test (Peng et al., 2009). In the last years, many studies have focused on noninvasive methods; *H. pylori* stool antigen test provides a simple alternative to the urea breath test and is appropriate for diagnosis and follow-up of infection (Gisbert and Pajares,

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PAPER II

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The Characteristics of *Helicobacter pylori* infection and Clinical Outcomes of Patient with Upper Gastrointestinal Bleeding Admitted at Hospital Universiti Sains Malaysia

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Abstract: Upper gastrointestinal bleeding (UGIB) remains one of the most common clinical life threatening emergencies which are associated with a high morbidity and mortality. The main aim of this study was to determine the cause of *Helicobacter pylori* (*H. pylori*) infection and the use of non-steroidal anti-inflammatory drugs (NSAID) in upper gastrointestinal bleeding patients. A retrospective record review study was conducted among UGIB confirmed patients from January 2009 and December 2012 at Hospital Universiti Sains Malaysia. All patients who were admitted in hospital were recruited. Data collection included age, gender, *Helicobacter pylori* positivity, associated symptoms and Endoscopic findings. There were 46 patients with a mean age of 62 years. *H. pylori* was detected only in 2 (4.3%) both in Male among UGIB patients by Campylobacter-like organism (CLO) test. The prevalence of UGIB was higher in men than women 27(58.7%). The most common cause of UGIB was peptic ulcer (56.5%) and especially high amongst male patients (59.2%).The second common cause of UGIB was gastritis (19.6%). The majority of the patients are NSAID users 25 (54.3%). In conclusion, Peptic ulcer disease is the leading cause of UGIB and mainly common among males and *H. pylori* infection in upper gastrointestinal bleeding patients was low.

Key words: *Helicobacter pylori* · Campylobacter-Like Organism Test · Gastritis · Peptic Ulcer · Upper Gastrointestinal Bleeding

INTRODUCTION

Upper gastrointestinal bleeding (UGIB) is a common medical emergency that requires hospitalization leading to higher patient morbidity and medical care [1]. The overall mortality rate associated with UGIB is nearly 10-15% [2]. The incidence rates of UGIB reveal a large geographic variation ranging from 100 to 150 cases per 100 000 population, with regular reports of higher incidences among men and elderly people [3, 4].

The most common cause of UGIB is Peptic ulcer bleeding [PUB], accounting for 31%-67% of all cases, followed by erosive disease, variceal bleeding, oesophagitis, malignancies and Mallory-Weis tears

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PAPER III

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Genotyping of *Helicobacter pylori* cagA Gene from a Patient Who Failed Eradication Therapy: A Case Report and Review of the Literature

Hussein Ali Osman[1], Habsah Hasan[2], Rapeah Suppian[2], Saravanan Arjunan[3], Zilfalil BA[3]

ABSTRACT

Helicobacter pylori (*H. pylori*) is a bacterium that causes chronic gastritis, gastric and duodenal ulcers and gastric cancer. Here we report a female patient presenting with dyspepsia. She was tested positive six times by the Rapid Urease test and Urea breath test (UBT). A culture of gastric biopsy was done and the isolate showed resistance to Clarithromycin and Metronidazole while polymerase chain reaction (PCR) revealed the presence of cagA *H. pylori* virulence gene. Presence of cagA might not be a risk factor in development of Metronidazole resistance to antibiotic therapy. In conclusion, we report a female Malaysian Indian with cagA positive H. pylori infection, but experienced Metronidazole resistance to antibiotic therapy.

KEY WORDS

Helicobacter pylori, CagA, antibiotic resistance

INTRODUCTION

Helicobacter pylori (*H. pylori*) is a Gram-negative microaerophilic bacterium that causes gastrointestinal diseases such as chronic gastritis, gastric and duodenal ulcers and gastric cancer[1-3]. *H. pylori* infection can be diagnosed by invasive (culture, rapid urease test, PCR and histology) and non invasive tests (serology, stool antigen test and ^{13}C-urea breath test (UBT)[4-6]. Invasive test has the advantage of being able to determine antibiotic- susceptibility and *H. pylori* genotypes[6].

In order to eradicate *H. pylori* infection, triple therapy using a proton pump inhibitor (PPI) with Clarithromycin and Amoxicillin or Metronidazole is recommended as the first-line treatment regimen. In case the triple therapy fails bismuth-containing quadruple therapy, which involves the inclusion of additional antibiotics to the first-line treatment regimen is used[6].

In Malaysia, Indians have been found to posses the highest prevalence of infection of about 68.9-75% as compared to Chinese 45.0-60.6% and Malays 8-43.3%[6-10].

A number of *H. pylori* virulence genes, including *cagA*, and *SabA* and have been associated with the most serious clinical outcomes and pathogenic bacteria[4,10].

CASE REPORT

This is 41 years old Malaysian female of Indian origin referred from Melaka Hospital for persistent dyspepsia. The patient was positive for *H. pylori* six times by Rapid urease test and UBT. The endoscopic finding was gastritis. She had a history of antiphospholipid syndrome, bronchial asthma and dysfunction uterine bleeding. Despite several treatment regimens the eradication therapy failed. Later on 20/2/2013 the patient underwent an upper gastrointestinal endoscopy at Hospital Kuala Lumpur and two gastric biopsies were taken for culture and sensitivity and Polymerase chain reaction (PCR).

The biopsy sample was put in brucella broth and immediately cultured onto Columbia agar supplemented with 8% sheep blood and dent

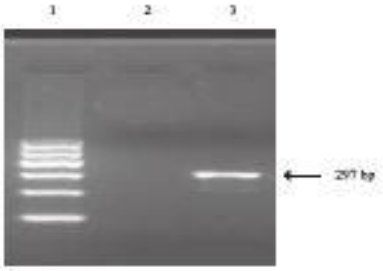

Figure 1. genotyping of cagA gene by PCR: lane 1, 100-bp DNA marker, lane 2 negative control without DNA, lane 3, *H. pylori* cagA positive strain.

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PAPER IV

Turkish Journal of Medical Sciences
http://journals.tubitak.gov.tr/medical/
Research Article

Turk J Med Sci
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Prevalence of *Helicobacter pylori cagA*, *babA2*, and *dupA* genotypes and correlation with clinical outcome in Malaysian patients with dyspepsia

Hussein Ali OSMAN[1], Habsah HASAN[1], Rapeah SUPPIAN[2], Syed HASSAN[3], Dzulkarnaen Zakaria ANDEE[3], Noortzan ABDUL MAJID[4], Bin-Alwi ZILFALIL[4,*]
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Received: 18.09.2014 • **Accepted/Published Online:** 20.12.2014 • **Printed:** 30.07.2015

Background/aim: The severity of disease outcome in dyspepsia has been attributed to *Helicobacter pylori* virulence genes. The aim of this study was to determine the distribution of *H. pylori* virulence genes (*cagA*, *babA2*, and *dupA*) and to determine whether or not there arises a significant correlation with clinical dyspepsia outcomes.

Materials and methods: *H. pylori* genotypes *cagA*, *babA2*, and *dupA* were identified by polymerase chain reactions from gastric biopsy samples in 105 *H. pylori*-positive patients.

Results: The positive rates for *cagA*, *babA2*, and *dupA* genes in *H. pylori* dyspeptic patients were 69.5%, 41.0%, and 22.9%, respectively. *cagA* was more prevalent in Indians (39.7%), *babA2* was more prevalent in Malays (39.5%), and *dupA* detection occurred more frequently in both Indians and Malays and at the same rate (37.5%). The Chinese inhabitants had the lowest prevalence of the three genes. Nonulcer disease patients had a significantly higher distribution of *cagA* (76.7%), *babA2* (74.4%), and *dupA* (75.0%). There was no apparent association between these virulence genes and the clinical outcomes.

Conclusion: The lower prevalence of these genes and variations among different ethnicities implies that the strains are geographically and ethnically dependent. None of the virulence genes were knowingly beneficial in predicting the clinical outcome of *H. pylori* infection in our subjects.

Key words: *Helicobacter pylori*, *cagA*, *babA2*, *dupA*, ethnicity, virulence genes

1. Introduction

Helicobacter pylori affects more than half of the world's population and over 70% of those inflicted reside in developing countries (1). *H. pylori* colonizes the gastric mucosa, causing chronic gastritis, peptic ulcers, gastric adenocarcinoma, and gastric mucosa-associated lymphoid tissue lymphoma (2,3). The clinical outcome linked to these diseases has been associated with host genetic factors, environmental factors, and pathogen virulence factors (4). A number of proteins, including vacA, cagA, babA, dupA, SabA, and iceA, have been inferred to play a vital role in the virulence of *H. pylori* by increasing the severity of the disease outcome (5-8).

The cytotoxin-associated gene (*cagA*) is most commonly associated with cytotoxin production and the induction of interleukin 8 (IL-8) by gastric epithelial cells (9). The cag pathogenicity island (PAI), in which the *cagA* gene is localized at one end, is involved in the induction of gastric IL-8 production, though most reports have demonstrated that the cagA protein is not involved in IL-8 induction (10,11). However, one study has verified that *cagA* participates in IL-8 induction in a strain-dependent and time-dependent manner (12). *cagA* is deemed to be one of the most imperative virulence factors in the pathogenesis of *H. pylori*. *cagA* belongs to a cag PAI that codes a type IV secretion system and this secretion system is responsible for the translocation of *cagA* into host cells (13). In Western countries, *cagA*-positive strains are reported to be linked with severe clinical outcomes, but in East Asian countries, it remains abstruse when trying to find this link because almost all *H. pylori* strains possess *cagA* (14).

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APPENDIX C: LIST OF ORAL AND POSTER PRESENTATION

ORAL PRESENTATION

1. **Hussein Ali Osman**, Habsah Hasan, Rapeah Suppian, Saravanan Arjunan , Amry AR, and Zilfalil BA. Prevalence of Helicobacter pylori cagA EPIYA motifs, ethnicity and clinical outcome in dyspeptic patients. GUT 2014 - Malaysian Society of Gastroenterology and Hepatology's Annual Scientific Meeting 22nd – 24th August 2014, Shangri-La Hotel, Kuala Lumpur
2. **Osman HA,** Habsah H, Rapeah S, Saravanan A, Nurzam SCH , Amry AR, S. Hassan, Andee DZ and Zilfalil BA. Association between Helicobacter pylori cagA gene and clinical outcome. 5th National Conference on Infectious Diseases (NCID). Renaissance Hotel, Kota Bharu, Kelantan, 25-26th August 2014
3. **AO, Hussein**, H Habsah, R Suppian, NA Che Hamzah, AR Amry, S Hassan, TS Sharifah Emilia and BA Zilfalil. Frequency of *Helicobacter pylori* in upper gastrointestinal bleeding patients in Hospital Universiti Sains Malaysia. Presentation at International Conference on Medical and Health Sciences (ICMHS)- 18th NCMHS-MSHG-12th ASMCPath , 22-24 May 2013, Kota Bharu, Kelantan, Malaysia.

POSTER PRESENTATION

1. **Hussein Ali Osman**, Habsah Hasan, Rapeah Suppian, Amry Abdul Rahim, Syed Hassan, Dzulkarnaen Zakaria Andee, Noorizan Abdul Majid and Zilfalil BA. Diversity of *Helicobacter pylori* cagA EPIYA motifs in different ethnic groups. Human Genome Meeting 2015. 14 – 17 March 2015,Kuala Lumpur Convention Centre, Malaysia
2. **HA Osman,** H Hasan, R Suppian, S Arjunan, SCH Nurzam, AR Amry, S Hassan, DZ Andee, HA Noorizan and BA Zilfalil. Distribution of Helicobacter pylori virulence genes in Malaysia. 15th Asia-Pacific Congress of Clinical Microbiology and Infection (15th APCCMI) Kuala Lumpur, Malaysia on the 26th - 29th November 2014.
3. **Hussein Ali Osman**, Habsah Hasan, Rapeah Suppian, Saravanan Arjunan , Amry Abdul Rahim, Syed Hassan , Dzulkarnaen Zakaria Andee Noorizan Abdul Majid and Bin- Alwi Zilfalil. Prevalence and endoscopic finding of *Helicobacter pylori* infection in dyspeptic patients. 19th National conference on medical and health sciences, 7-8th September 2014, school of dental sciences, Kubang Kerian, Kelantan.
4. **Hussein Ali Osman**, Habsah Hasan, Rapeah Suppian, Shashi Kumar Menon, Norhaniza binti Bahar, Nurzam SCH and BA Zilfalil. Diagnosis of *Helicobacter pylori* Infection by new stool antigen test in dyspeptic patients. International Congress of the Malaysian Society for Microbiology 2013 (ICMSM2013) , 12-15 December 2013 Langkawi Lagoon Resort, Malaysia.

APPENDIX D: STUDY APPROVAL

UNIVERSITI SAINS MALAYSIA

Jawatankuasa Etika Penyelidikan Manusia USM (JEPeM)
Human Research Ethics Committee USM (HREC)

Our. Ref. : USMKK/PPP/JEPeM [247.3.(17)]
Date : 27th March 2012

Universiti Sains Malaysia
Kampus Kesihatan,
16150 Kubang Kerian,
Kelantan. Malaysia.
T: 609 - 767 3000 *samb.* 2350 / 2352
F: 609 - 767 2351
E: jepem@kk.usm.my
www.crp.kk.usm.my

Mr. Hussein Ali Osman
Department of Medical Microbiology and Parasitology
School of Medical Sciences
Universiti Sains Malaysia
16150 Kubang Kerian, Kelantan.

The Human Research Ethics Committee, Universiti Sains Malaysia (FWA Reg. No: 00007718; IRB Reg. No: 00004494) has approved in principle the study mentioned below:

Title	The Role of Specific Helicobacter Pylori Virulence and Host Genetic Factors in Gastroduodenal Disease.		
Protocol No	-	**Principle Investigator**	Mr. Hussein Ali Osman
Date of approval Protocol received Reviewed by Committee Received Amended Protocol	27th March 2012 22nd February 2012 27th February 2012 26th March 2012	**Co-Investigator(s)**	Prof. Dr. Zilfalil Alwi Assoc. Prof. Dr. Habsah Hasan Dr. Rapeah Suppian
Research Center	Department of Medical Microbiology and Parasitology and Central Research Laboratory, School of Medical Sciences, Universiti Sains Malaysia.	**Date of study start**	April 2012 – March 2015
Financial Support	-	**Number of Samples**	262 samples

The following item (✓) have been received and reviewed:-

(✓) **Ethical Approval Application Form**
(✓) **Study Protocol**
(✓) **Patient Information Sheet and Consent Form**
(✓) **Data Collection Form**

Investigator(s) are required to:
a) follow instructions, guidelines and requirements of the Human Research Ethics Committee, Universiti Sains Malaysia (JEPeM)
b) report any protocol deviations/violations to Human Research Ethics Committee (JEPeM)
c) comply with International Conference on Harmonization – Guidelines for Good Clinical Practice (ICH-GCP)
d) note that Human Research Ethics Committee (JEPeM) may audit the approved study.

PROFESSOR DR. MOHD SHUKRI OTHMAN
Chairman
Human Research Ethics Committee

JAWATANKUASA ETIKA & PENYELIDIKAN PERUBATAN
(Medical Research & Ethics Committee)
KEMENTERIAN KESIHATAN MALAYSIA
d/a Institut Pengurusan Kesihatan
Jalan Rumah Sakit, Bangsar
59000 Kuala Lumpur

Tel. : 03 2282 9082/03 2282 9085
03 2287 4032/03 2282 0491
Faks : 03 2287 4030

Ruj : (5)dlm.KKM/NIHSEC/P12-421
Tarikh: 22 May 2014

ENCIK HUSSEIN ALI OSMAN
JABATAN MIKROBIOLOGI & PARASITOLOGI,
UNIVERSITI SAINS MALAYSIA,
KUBANG KERIAN,
16150 KOTA BHARU,
KELANTAN,

Per: Annual Ethical Renewal for 2014

NMRR-12-358-11418
THE ROLE OF SPECIFIC HELICOBACTER PYLORI (HP) VIRULENCE AND HOST GENETIC FACTORS IN GASTRODUODENAL DISEASE

With reference to the 'Continuing Review Form' submitted/ dated 18 June 2013, we are pleased to inform that the conduct of the above study has been granted approval for a year by the Medical Research & Ethics Comittee, Ministry of Health Malaysia. Please note that the approval is valid until 18 June 2014. To renew the approval, a completed 'Continuing Review Form' has to be submitted to MREC at least 2 months before the expiry of the approval.

The MREC, Ministry of Health Malaysia operates in accordance to the International Harmonization Good Clinical Practice Guidelines.

Thank you.

"BERKHIDMAT UNTUK NEGARA"

Yours sincerely,

.....
(DATO' DR CHANG KIAN MENG)
Chairman
Medical Research & Ethics Committee
Ministry of Health Malaysia

PEJABAT TIMBALAN KETUA PENGARAH KESIHATAN
OFFICE OF THE DEPUTY DIRECTOR-GENERAL OF HEALTH
(PENYELIDIKAN & SOKONGAN TEKNIKAL)
(RESEARCH & TECHNICAL SUPPORT)
KEMENTERIAN KESIHATAN MALAYSIA
MINISTRY OF HEALTH MALAYSIA
Aras 12, Blok E7, Parsel E, Presint 1
Level 12, Block E7, Parcel E, Precint 1
Pusat Pentadbiran Kerajaan Persekutuan
Federal Government Administrative Centre
62590 PUTRAJAYA

Tel. : 03-88832543
Faks : 03-88895184

JAWATANKUASA ETIKA & PENYELIDIKAN PERUBATAN
KEMENTERIAN KESIHATAN MALAYSIA
d/a Institut Pengurusan Kesihatan
Jalan Rumah Sakit, Bangsar
59000 Kuala Lumpur

Ruj. Kami :(2) dlm.KKM/NIHSEC/08/0804/P12-421
Tarikh : 30 Julai 2012

Encik Hussein Ali Osman
Jabatan Mikrobiologi & Parasitologi
Universiti Sains Malaysia, Kampus Kubang Kerian

Tuan,

NMRR-12-358-11418
The Role of Specific Helicobacter Pylori (HP) Virulence and Host Genetic Factors in Gastroduodenal Disease

Lokasi Projek : Hospital Kuala Lumpur / Hospital Tengku Ampuan Afzan

Dengan hormatnya perkara di atas adalah dirujuk.

2. Jawatankuasa Etika & Penyelidikan Perubatan (JEPP), Kementerian Kesihatan Malaysia (KKM) mengambil maklum bahawa projek tersebut adalah untuk memenuhi keperluan kajian program PhD Mikrobiologi, Universiti Sains Malaysia.

3. Sehubungan dengan ini, dimaklumkan bahawa pihak JEPP KKM tiada halangan, dari segi etika, ke atas pelaksanaan projek tersebut. JEPP mengambil maklum bahawa kajian ini tidak melibatkan sebarang intervensi dan hanya menggunakan sampel tisu, darah dan tinja untuk mengumpul data. Segala rekod dan data pegawai adalah SULIT dan hanya digunakan untuk tujuan kajian dan semua isu serta prosedur mengenai *data confidentiality* mesti dipatuhi. Kebenaran daripada Pengarah Hospital/ Kesihatan Negeri di mana kajian akan dijalankan mesti diperolehi terlebih dahulu sebelum kajian dijalankan. Tuan perlu akur dan mematuhi keputusan tersebut.

4. Adalah dimaklumkan bahawa kelulusan ini adalah selama setahun dan tuan perlu menghantar *'Continuing Review Form'* pada bulan November setiap tahun bagi memperbaharui kelulusan etika. Laporan tamat kajian dan sebarang penerbitan dari kajian ini hendaklah dikemukakan kepada Jawatankuasa Etika & Penyelidikan Perubatan selepas tamatnya kajian ini.

Sekian terima kasih.

BERKHIDMAT UNTUK NEGARA

Saya yang menurut perintah,

(DATO' DR CHANG KIAN MENG)
Pengerusi
Jawatankuasa Etika & Penyelidikan Perubatan
Kementerian Kesihatan Malaysia

Appendix E: Data collection form (Data sheet questionnaire Malay& English)

Kajian Helikobakter pylori 2013
Helicobacter pylori study 2013

No Kes / Case No:

No PendaftaranHospital / Hospital No:

Umur / Age:

No IC:

Kumpulan Etnik / Ethnic group:	Malay	Indian	Chinese

Jantina / Sex:	Lelaki / Male	Perempuan / Female

Generasi / Generation:	Pertama / First	Kedua / Second	Ketiga / Third

Pengaduan terlapor / presenting complaint:

Ketidakselesaan Perut / Abdominal discomfort	Ya / Yes	Tidak/ No
Pedih hulu hati / Heart burn	Ya / Yes	Tidak / No
Loya / Muntah / Nausea / vomiting	Ya / Yes	Tidak / No
Sakit disebabkan makanan/ pain related to meals	Ya / Yes	Tidak / No

1

Sejarah Sosial / Social history

Merokok / Smoking	Sekarang / Current	Sebelum / Previous	Tidak Pernah / Never
Alkohol / Alcohol	Sekarang / Current	Sebelum / Previous	Tidak Pernah / Never
Kediaman sekarang / Current residency	Bandar / Urban	Luar Bandar / Rural area	

Pendidikan / Education	Tiada/ None	Menengah/ Secondary	Diploma/ College	Universiti/ University	Sarjana/ Postgraduate

Pekerjaan / Occupation	Tidak Bekerja / Unemployed	Buruh /Bekerja sendiri/ Manual	Pentadbir / Administration	Profesional / Professional

Makanan Masin / Salty food	Sikit / Low	Sederhana / Moderate	Banyak / Severe
Makanan Berempah / Spicy food	Sikit / Low	Sederhana / Moderate	Banyak / Severe
Diet	Bukan vegetarian / Non-vegetarian	Vegetarian / Vegetarian	
Bekalan Air Bersih / Water supply	Air Paip / Tap water	Air perigi / Well water	
Pembuangan sisa / Sewage disposal	Ya / Yes	Tidak / No	

Sejarah Perubatan / Medical history

Penyakit Jantung Iskemia (IHD) / Ischaemic heart disease	Ya / Yes	Tidak / No
Asthma	Ya / Yes	Tidak / No
Batuk Kering (TB) / Tuberculosis	Ya / Yes	Tidak / No
Diabetes	Ya / Yes	Tidak / No

Lain-lain / others .....

Pengubatan Semasa / Current medication

Ya / Yes	Tidak / No

Antasid / Antacid

Ya / Yes	Tidak / No

.....

PPI

Ya / Yes	Tidak / No

.....

Lain-lain / Others

Ya / Yes	Tidak / No

.....

.....

OGD Findings:

Gastritis: | Yes | No | Location.....

Gastric ulcer: | Yes | No | Location.....

Gastric erosion: | Yes | No | Location.....

Duodenal ulcer: | Yes | No | Location.....

Duodenitis: | Yes | No | Location.....

Gastric cancer: | Yes | No | Location.....

Other findings:.....

APPENDIX F: PATIENTS INFORMATION CONSENT FORM (ENGLISH A AND MALAY B LANGUAGES)

JAWATANKUASA ETIKA PENYELIDIKAN (MANUSIA) - JEPeM

RESEARCH ETHICS COMMITTEE (HUMAN)

BORANG MAKLUMAT DAN KEIZINAN PESAKIT/ SUBJEK

PATIENT INFORMATION AND CONSENT FORM

(PROJEK PENYELIDIKAN)

(RESEARCH PROJECT)

Borang Maklumat dan Keizinan Pesakit/Subjek ***yang digunakan dalam Projek Penyelidikan mestilah mengikuti format maklumat berikut:***
The Patient Infomation and Consent Form **used in the Research Project must be according to these information formats:**

- ***Tajuk Kajian* / Topic of the Research**
- ***Pengenalan* / Introduction**
- ***Tujuan Kajian* / Purpose of the Study**
- ***Kelayakan Penyertaan* / Qualification to Participate**
- ***Prosedur-prosedur Kajian* / Study Procedures**
- ***Risiko* / Risks**
- ***Melaporkan Pengalaman Kesihatan* / Reporting Health Experiences**
- ***Penyertaan dalam Kajian* / Participation in the Study**
- ***Manafaat yang Mungkin Diperolehi* / Possible Benefits**
- ***Soalan* / Questions**
- ***Kerahsiaan* / Confidentiality**
- ***Tandatangan* / Signatures**

Sebagai** CONTOH**, sila rujuk Borang Maklumat dan Keizinan Pesakit yang dilampirkan.
As an EXAMPLE**, please refer to the attached Patient Infomation and Consent Form.**
(Versi Bahasa Malaysia) / (Bahasa Malaysia Version)

1. *LAMPIRAN A*
   <TAJUK KAJIAN>
2. *LAMPIRAN S (Borang Keizinan Pesakit)*
3. *LAMPIRAN G (Borang Keizinan Pesakit* – Sampel Genetik*)*
4. *LAMPIRAN P (Borang Keizinan Penerbitan Bahan yang Berkaitan dengan Subjek)*

(Versi Bahasa Inggeris) / (English Version)

1. *ATTACHMENT B*
   <RESEARCH TITLE>
2. *ATTACHMENT S (Patient Information and Consent Form)*
3. *ATTACHMENT G (Patient Information and Consent Form* – Genetic Sample*)*
4. *ATTACHMENT P (Subject's Material Publication Consent Form)*

ATTACHMENT B

RESEARCH INFORMATION

Research Title:

The role of specific *helicobacter pylori* virulence and host genetic susceptible factors in gastroduodenal disease

Researcher's Name: Hussein Ali Osman

MMC Registration No. :

INTRODUCTION

Helicobacter pylorus (H. pylori) is a spiral gram-negative microaerophilic bacillus. This bacteria is one of the most common worldwide human pathogens, it is present in at least 50% of the world's population, with the highest incidence recorded in industrially underdeveloped areas, including Asia, Africa and South America. *H. pylori* colonizes the human stomach and persists for several decades, causing chronic gastritis and peptic ulcer diseases. Studies have suggested that chronic infection by *H. pylori* is an important risk factor for the development of gastric carcinoma.

You are invited to take part voluntarily in the research by filling the data sheet provided to you. This data sheet is performed to identify the prevalence and role of *caga, dupA,babA* and *sabA* genotypes of *Helicobacter pylori* and DNA variants in gastroduodenal diseases.

Before you agree to participate in this research study, it is important that you read and understand this form. If you decide to participate, you will receive a copy of this form for your records.
There is no need to write your name or identity card number on the questionnaire and all information provided will be kept confidential.

PURPOSE OF THE STUDY

This study is important because it will determine the sensitivity and specificity of a monoclonal stool antigen test, find out if bacterial *H. Pylori* virulence factors (*cagA*, *dupA*, *babA* and *sabA*) and specific human genetic factors play a role in determining the severity of gastro duodenal diseases

QUALIFICATION TO PARTICIPATE

The researcher will discuss with you about the criteria to join the research based on your medical history. It is important that you are completely truthful with all the informations given. You are not allowed to participate in this study if you do not meet all requirements**.**

INCLUSION AND EXCLUSION CRITERIA FOR THIS STUDY:

Inclusion criteria

- 18 years and above
- Patients who are positive for *H.pylori*
- Patient with persistent abdominal pain, heartburn, acid regurgitation, sucking sensation, nausea and vomiting.
- Discomfort over the preceding 3-month period.
- Not been using nonstreroidal anti-inflammatory drugs (NSAID), antacids or any antibiotics within the previous two weeks.
- Willing to participate in the study.

Exclusion criteria

- Less than 18years
- Refused to give informed consent
- Patients with past history of *H. pylori* eradication therapy
- Patients taking antibiotics, H2-receptor blockers, bismuth or proton pump inhibitors in the preceding.

STUDY PROCEDURES

The study will be carried from the informed and consenting adult patients with gastro duodenal disease who will be referred to the Endoscopy Unit, Hospital Universiti Sains Malaysia and other Hospitals. In phase one of the studies, during upper endoscopy four antral biopsies will be taken from each patient. This will be used for Invasive tests (Campylobacter like organism (CLO test), culture, histology), and DNA extraction for PCR to amplify *cagA, dupA, babA* and *SabA* while stool sample will be collected for Non-invasive tests (stool antigen test). In phase two blood samples will be collected and DNA microarray analysis will be employed to identify the responsible genes in the host genome. Analysis of genomic variability, biological pathways and genes involved in determining the severity of gastro duodenal diseases will be investigated.

RISKS

The participant will not be exposed to any medical risks in this study. If any important information will be found during the study that may influence the participant's agreement, participant will be informed immediately.

REPORTING HEALTH EXPERIENCES

If you have any injury, bad effect, or any other unusual health experience during this study, make sure that you immediately inform Mr Hussein Ali Osman (H/p .01116513040), Department of Medical Microbiology and Parasitology, School of Medical Sciences, USM, Health Campus or you can call him at anytime, day or night, to report such health experiences.

PARTICIPATION IN THE STUDY

Your taking part in this study is entirely voluntary. Participant may refuse to take part in the study or stop his/her participation in the study at anytime, without a penalty or loss of benefits to which you are otherwise entitled. Your participation may also be stopped anytime by the researcher or sponsor without your consent.

POSSIBLE BENEFITS (Benefit to Individual, Community and University)

You may receive information about your health from the findings of this study. Information obtained from this research will benefit you to get proper treatment or new strategy to reduce the incidence of *H.pylori*. It will also benefit other patients in future. This study will also help in the identification of any novel gene susceptible to *H.pylori* infection

QUESTIONS

If you have any question about this study or your rights, please contact;

Hussein Ali Osman

Department of Medical Microbiology and Parasitology

School of Medical Sciences

USM Health Campus, Kubang Kerian, Kelantan.

Contact No. H/P 01116513040

If you have any questions regarding the Ethical Approval or any issue/problem related to this study, please contact;

Puan Mazlita Zainal Abidin

Secretary of Research Ethics Committee (Human) USM

Clinical Sciences Research Platform

USM Health Campus

Tel. No. : 09-767 2355 / 09-767 2352

Email: jepem@kk.usm.my

CONFIDENTIALITY

Your medical information will be kept confidential by the researcher and staff and will not be made publicly available unless disclosure is required by law.

Data obtained from this study that does not identify you individually will be published for knowledge purposes.

Your original medical records may be reviewed by the researcher, the Ethical Review Board for this study, and regulatory authorities for the purpose of verifying clinical trial procedures and/or data. Your medical information may be held and processed on a computer.

By signing this consent form, you authorize the record review, information storage and data transfer described above.

SIGNATURES

To be included in the study, you or a legal representative must sign and date the signature page [ATTACHMENT S or ATTACHMENT G (for genetic sample only) or ATTACHMENT P]

ATTACHMENT S

Patient/Subject Information and Consent Form

(Signature Page)

Research Title: **The role of specific helicobacter pylori virulence and host genetic susceptible factors in gastroduodenal disease**

Researcher's Name: **Hussein Ali Osman** _____

To become a part this study, you or your legal representative must sign this page. By signing this page, I am confirming the following:

- I have read all of the information in this Patient Information and Consent Form including any information regarding the risk in this study and I have had time to think about it.
- All of my questions have been answered to my satisfaction.
- I voluntarily agree to be part of this research study, to follow the study procedures, and to provide necessary information to the doctor, nurses, or other staff members, as requested.
- I may freely choose to stop being a part of this study at anytime.
- I have received a copy of this Patient Information and Consent Form to keep for myself.

_____ _____

Patient Name **(Print or type)** Patient Initials **and** Number

_____ _____

Patient I.C **No. (New)** Patient I.C **No. (Old)**

_____ _____

Signature of Patient **or Legal Representative** Date **(dd/MM/yy)**

_____ **(Add time if applicable)**

Name of Individual

Conducting Consent Discussion (Print or Type)

_____ _____

Signature of Individual **(dd/MM/yy)** Date

Conducting Consent Discussion _____ _____

Name & Signature of Witness Date **(dd/MM/yy)**

Note: **i)** **All subject/patients who are involved in this study will not be covered by insurance.**

ATTACHMENT G

Patient/ Subject Information and Consent Form

(Signature Page)

Research Title: **The role of specific *helicobacter pylori* virulence and host genetic susceptible factors in gastroduodenal disease**

Researcher's Name: **Hussein Ali Osman**

To become a part this study, you or your legal representative must sign this page. By signing this page, I am confirming the following:

- I have read all of the information in this Patient Information and Consent Form including any information regarding the risk in this study and I have had time to think about it.
- All of my questions have been answered to my satisfaction.
- I voluntarily agree to be part of this research study, to follow the study procedures, and to provide necessary information to the doctor, nurses, or other staff members, as requested.
- I may freely choose to stop being a part of this study at anytime.
- I have received a copy of this Patient Information and Consent Form to keep for myself.

Patient Name **(Print or type)** Patient Initials **and** Number

Patient I.C **No.** (New) Patient I.C **No.** (Old**)**

Signature of patient **or Legal Representative** Date **(dd/MM/yy)**

(Add time if applicable)

Name of Individual

~~conducting Consent Discussion (Print or Type~~

Signature of Individual Date **(dd/MM/yy)**

~~Conducting Consent Discussion~~

Name & Signature of Witness Date **(dd/MM/yy)**

Note: **i)** **All subject/patients who are involved in this study will not be covered by insurance.**

ii) **Excess samples from this research will not be used for other reasons and will be destroyed with the consent from the Research Ethics Committee (Human), USM.**

ATTACHMENT P

Patient's Material Publication Consent Form

Signature Page

Research Title: **The role of specific helicobacter pylori virulence and host genetic susceptible factors in gastroduodenal disease**

Researcher's Name: **Hussein Ali Osman**

To become a part this study, you or your legal representative must sign this page.

By signing this page, I am confirming the following:

- I understand that my name will not appear on the materials published and there will be efforts to make sure that my name is kept confidential although the confidentiality is not completely guaranteed due to unexpected circumstances.
- I have read the materials or general description of what the material contains and reviewed all photographs and figures in which I am included that could be published.
- I have been offered the opportunity to read the manuscript and to see all materials in which I am included, but have waived my right to do so.
- All the published materials will be shared among the medical practitioners, scientists and journalist worldwide.
- The materials will also be used in local publications, book publications and accessed by many local and international doctors worldwide.
- I hereby agree and allow the materials to be used in other publications required by other publishers with these conditions:
- The materials will not be used as advertisement purposes nor as packaging materials.
- The materials will not be used out of context – i.e., sample pictures will not be used in an article which is unrelated to the subject.

Patient Name **(Print or type)**	Patient Initials **or** Number
Patient I.C No.	**Patient's Signature** Date **(dd/MM/yy)**
Name and Signature of Individual	Date **(dd/MM/yy)**

Conducting Consent Discussion

Note: **i)** **All subject/patients who are involved in this study will not**

BORANG MAKLUMAT DAN KEIZINAN PESAKIT

PATIENT INFORMATION AND CONSENT FORM

(PROJEK PENYELIDIKAN)

(RESEARCH PROJECT)

Borang Maklumat dan Keizinan Pesakit ***yang digunakan dalam Projek Penyelidikan mestilah mengandungi maklumat-maklumat berikut:***
The Patient Infomation and Consent Form **used in the Research Project must have these information:**

- ***Tajuk Kajian* / Topic of the Research**
- ***Pengenalan* / Introduction**
- ***Tujuan Kajian* / Purpose of the Study**
- ***Kelayakan Penyertaan* / Qualification to Participate**
- ***Prosedur-prosedur Kajian* / Study Procedures**
- ***Risiko* / Risks**
- ***Melaporkan Pengalaman Kesihatan* / Reporting Health Experiences**
- ***Rawatan Lain* / Other Treatments**
- ***Penyertaan dalam Kajian* / Participation in the Study**
- ***Pampasan dan Rawatan untuk Kecederaan* / Treatment and Compensation for Injury**
- ***Munafaat yang Mungkin* / Possible Benefits**
- ***Bayaran Doktor (Penyelidik)* / Investigator's Payment**
- ***Soalan* / Questions**
- ***Kerahsiaan* / Confidentiality**
- ***Tandatangan* / Signatures**

Sebagai *CONTOH****, sila rujuk Borang Maklumat dan Keizinan Pesakit yang dilampirkan.***
As an EXAMPLE**, please refer the attached Patient Infomation and Consent Form.**
(Versi Bahasa Malaysia) / (Bahasa Malaysia Version)

5. *LAMPIRAN A*
   "Peranan khusus kevirulenan helicobacter pylori dan faktor genetik perumah dalam penyakit gastroduodenal"
6. *LAMPIRAN S (Borang Keizinan Pesakit)*

7. *LAMPIRAN G (Borang Keizinan Pesakit* – Sampel Genetik*)*

8. *LAMPIRAN P (Borang Keizinan Penerbitan Bahan yang Berkaitan dengan Peserta)*

(Versi Bahasa Inggeris) / (English Version)

5. *LAMPIRAN B*
   "Peranan khusus kevirulenan helicobacter pylori dan faktor genetik perumah dalam penyakit gastroduodenal"

6. *ATTACHMENT S (Patient Information and Consent Form)*

7. *ATTACHMENT G (Patient Information and Consent Form* – Genetic Sample*)*

8. *ATTACHMENT P (Patient's Material Publication Consent Form)*

LAMPIRAN

B

MAKLUMAT KAJIAN

Tajuk Kajian: ***Peranan khusus kevirulenan Helicobacter pylori dan faktor genetik perumah dalam penyakit gastroduodenal***

Nama Penyelidik: Hussein Ali Osman

Pengenalan

Anda dipelawa untuk menyertai satu kajian penyelidikan secara sukarela dengnan menjawab soalan soal selidik yang diberikan kepada anda. Soal selidik ini diedarkan untuk mengenal pasti prevalen dan peranan genotaip helicobacter pylori* caga,dupA,babA dan sabA *serta varian DNA dalam penyakit gastroduodenal.

Sebelum anda bersetuju untuk menyertai kajian penyelidikan ini, adalah penting anda membaca dan memahami borang ini. Sekiranya anda menyertai kajian ini, anda akan menerima satu salinan borang ini untuk disimpan sebagai rekod anda.

Tujuan Kajian

Kajian ini penting kerana ia akan memberi peluang kepada saintis dan para klinisian untuk mengetahui samada faktor bakteria dan faktor genetik perumah (SNPs dan CNVs) memainkan peranan dalam kerentanan dan keparahan penyakit gastro duodenal

Kelayakan Penyertaan

Penyelidik yang bertanggungjawab dalam kajian ini akan membincangkan kriteria penyertaan berdasarkan sejarah perubatan anda. Adalah penting anda berterus terang dengan doktor tentang maklumat yang diberikan. Anda tidak seharusnya menyertai kajian ini sekiranya anda tidak memenuhi semua syarat kelayakan.

Kriteria penyertaan dan pengecualian untuk kajian ini:

Kriteria penyertaan

- **18 tahun keatas**
- **Pesakit yang mengalami sakit perut berterusan, pedih ulu hati, regurgitasi asid, sensasi menyedut, loya dan muntah.**
- **Ketidakselesaan dalam tempoh 3 bulan sebelum**
- **Tidak menggunakan ubat anti inflamasi tanpa steroid, antasid atau antibiotik dalam tempoh dua minggu sebelumnya.**
- **Bersetuju menyertai kajian ini.**

Kriteria pengecualian

- **Kurang daripada 18 tahun**
- **Tidak memberi persetujuan**
- **Pesakit dengan sejarah terapi pembasmian *H.pylori***
- **Pesakit yang mengambil antibiotik, penghalang reseptor-H2, bismut atau pam perencat proton sebelumnya**

Prosedur- Kajian
Kajian akan dijalankan keatas pesakit dewasa yang mengalami penyakit gasroduodenum dan telah memberikan persetujuan. Pesakit-pesakit ini akan dirujuk kepada Unit Endoskopi, Hospital Universiti Sains Malaysia. Dalam fasa pertama kajian, empat biopsi antral semasa endoskopi atas akan diambil daripada setiap pesakit. Ia kemudiannya akan digunakan untuk ujian invasif (Campylobacter like organism(CLO test), kultur ,histologi).

Ekstrasi DNA akan dibuat untuk PCR bagi menguatkan *cagA,dupA, babA* dan *SabA*, sementara itu sampel najis akan diambil untuk ujian tak-invasif (ujian antigen najis). Dalam fasa kedua analisis tatasusunanmikro akan digunakan untukmengenalpasti gen yang bertanggungjawab dalam genom perumah.Analisi kebolehubahan genom, laluan biologikal dan gen yang terlibat dalam menentukan keparahan penyakit gastro duodenal akan dikaji.

Risiko

Peserta tidak akan didedahkan kepada apa-apa risiko perubatan semasa kajian ini dijalankan. Jika terdapat maklumat semasa kajian yang akan mempengaruhi perjanjian perseta, peserta akan dimaklumkan segera.

Melaporkan Pengalaman Kesihatan

Jika anda mengalami apa-apa kecederaan, kesan buruk, atau apa-apa pengalaman kesihatan yang luarbiasa semasa kajian ini, pastikan anda memberitahu En. Hussein Ali Osman di [No. Telefon 01116513040)] jabatan Perbatan Mikrobiologi dan Parasitologi, Pusat Pengajian Sains Perubatan, USM, kampus Kesihatan atau anda boleh. membuat panggilan pada bila-bila masa, siang atau malam, untuk melaporkan pengalaman sedemikian.

Penyertaan Dalam Kajian

Penyertaan anda dalam kajian ini adalah secara sukarela. Anda boleh menolak penyertaan dalam kajian ini atau anda boleh menamatkan penyertaan anda dalam kajian ini pada bila-bila masa, tanpa sebarang hukuman atau kehilangan sebarang manfaat yang sepatutnya diperolehi oleh anda.Penyertaan anda mungkin juga diberhentikan oleh doktor kajian atau pihak penaja tanpa persetujuan anda.

Manfaat yang Mungkin

Anda mungkin menerima maklumat tentang kesihatan anda daripada keputusan kajian ini. Maklumat yang diperolehi daripada kajian ini akan memberi manfaat kepada anda untuk mendapatkan rawatan yang lebih baik atau strategi baru untuk mengurangkan insiden *H. pylori*. Ia juga akan memanfaatkan pesakit lain dimasa hadapan. Kajian ini juga kan membantu dalam mengenalpasti gen tunggal yang mudah dipengaruhi oleh jangkitan *H.pylori*.

Soalan

Sekiranya anda mempunyai sebarang soalan mengenai prosedur kajian ini atau hak-hak anda, sila hubungi;

Hussein Ali Osman

Jabatan Perubatan Mikrobiologi dan Parasitologi

Pusat Pengajian sains Perubatan

USM Kampus Kesihatan.

Tel: 01116513040

Email: jepem@kk.usm.my

Sekiranya anda mempunyai sebarang soalan berkaitan kelulusan Etika kajian ini, sila hubungi;

Puan Mazlita Zainal Abidin

Setiausaha Jawatankuasa Etika Penyelidikan (Manusia) USM

Pelantar Penyelidikan Sains Klinikal, USM Kampus Kesihatan.

No. Tel: 09-7672355 / 09-7672352

Email: jepem@kk.usm.my

Kerahsiaan

Maklumat perubatan anda akan dirahsiakan oleh doktor dan kakitangan kajian dan tidak akan dedahkan secara umum melainkan jika ia dikehendaki oleh undang-undang.

Data yang diperolehi dari kajian yang tidak mengenalpasti anda secara perseorangan akan diberi kepada pihak penaja dan/atau wakil-wakilnya dan mungkin akan diterbitkan untuk kajian selanjutnya dalam bidang ini di HUSM.

Rekod perubatan anda yang asal mungkin akan dilihat oleh pihak penaja dan/atau wakil-wakilnya, Lembaga Etika kajian ini dan pihak berkuasa regulatori untuk tujuan mengesahkan prosedur dan/atau data kajian klinikal. Maklumat perubatan anda mungkin akan disimpan dalam komputer dan diproses dengannya.

Dengan menandatangani borang persetujuan ini, anda membenarkan penelitian rekod, penyimpanan maklumat dan pemindahan data seperti yang dihuraikan di atas.

Tandatangan

Untuk dimasukkan ke dalam kajian ini, anda atau wakil sah anda mesti menandatangani serta mencatatkan tarikh halaman tandatangan (LAMPIRAN S **atau** LAMPIRAN G (untuk sampel genetik) **atau** LAMPIRAN P**).**

LAMPIRAN S

Borang Keizinan Pesakit

(Halaman Tandatangan)

Tajuk Kajian: ***Peranan khusus kevirulenan Helicobacter pylori dan faktor genetik perumah dalam penyakit gastroduodenal***

Nama Penyelidik: ***Hussein Ali Osman***

Untuk menyertai kajian ini, anda atau wakil sah anda mesti menandatangani mukasurat ini. Dengan menandatangani mukasurat ini, saya mengesahkan yang berikut:

- **Saya telah membaca semua maklumat dalam Borang Maklumat dan Keizinan Pesakit ini** termasuk apa-apa maklumat berkaitan risiko yang ada dalam kajian **dan saya telah pun diberi masa yang mencukupi untuk mempertimbangkan maklumat tersebut.**
- **Semua soalan-soalan saya telah dijawab dengan memuaskan.**
- **Saya, secara sukarela, bersetuju menyertai kajian penyelidikan ini, mematuhi segala prosedur kajian dan memberi maklumat yang diperlukan kepada doktor, para jururawat dan juga kakitangan lain yang berkaitan apabila diminta.**
- **Saya boleh menamatkan penyertaan saya dalam kajian ini pada bila-bila masa.**
- **Saya telah pun menerima satu salinan Borang Maklumat dan Keizinan Pesakit untuk simpanan peribadi saya.**

Nama Pesakit **(Dicetak atau Ditaip)**	Nama Singkatan & No. Pesakit
No. Kad Pengenalan Pesakit **(Baru)**	No. K/P **(Lama)**
Tandatangan Pesakit ***atau Wakil Sah***	*Tarikh* ***(dd/MM/yy)*** ***(Masa jika perlu)***
Nama & Tandatangan Individu **yang Mengendalikan**	Tarikh **(dd/MM/yy)**
Perbincangan Keizinan (Dicetak atau Ditaip)	
Nama Saksi dan Tandatangan	Tarikh **(dd/MM/yy)**

Nota: **i) Semua subjek/pesakit yang mengambil bahagian dalam projek penyelidikan ini tidak dilindungi insuran.**

LAMPIRAN G

Borang Keizinan Pesakit untuk *Sampel Genetik*

(Halaman Tandatangan)

Tajuk Kajian: ***Peranan khusus kevirulenan Helicobacter pylori dan faktor genetik perumah dalam penyakit gastroduodenal*** Nama Penyelidik: *Hussein Ali Osman*

Untuk menyertai kajian ini, anda atau wakil sah anda mesti menandatangani mukasurat ini. Dengan menandatangani mukasurat ini, saya mengesahkan yang berikut:

- **Saya telah membaca semua maklumat dalam Borang Maklumat dan Keizinan Pesakit ini** termasuk apa-apa maklumat berkaitan risiko yang ada dalam kajian **dan saya telah pun diberi masa yang mencukupi untuk mempertimbangkan maklumat tersebut.**
- **Semua soalan-soalan saya telah dijawab dengan memuaskan.**
- **Saya, secara sukarela, bersetuju menyertai kajian penyelidikan ini, mematuhi segala prosedur kajian dan memberi maklumat yang diperlukan kepada doktor, para jururawat dan juga kakitangan lain yang berkaitan apabila diminta.**
- **Saya boleh menamatkan penyertaan saya dalam kajian ini pada bila-bila masa.**
- **Saya telah pun menerima satu salinan Borang Maklumat dan Keizinan Pesakit untuk simpanan peribadi saya.**

Nama Pesakit **(Dicetak atau Ditaip)**	Nama Singkatan & No. Pesakit
No. Kad Pengenalan Pesakit **(Baru)**	No. K/P **(Lama)**
Tandatangan Pesakit ***atau Wakil Sah***	*Tarikh* ***(dd/MM/yy)*** ***Masa (jika perlu)***
Nama & Tandatangan Individu **yang Mengendalikan Perbincangan Keizinan (Dicetak atau Ditaip)**	Tarikh **(dd/MM/yy)**
Nama Saksi dan Tandatangan	Tarikh **(dd/MM/yy)**

Nota: i) **Lebihan sampel kajian ini akan dilupuskan dan tidak akan digunakan untuk tujuan lain kecuali setelah mendapat**

kebenaran daripada Jawatankuasa Etika Penyelidikan (Manusia), USM.

ii) Semua subjek/pesakit yang mengambil bahagian dalam projek penyelidikan ini tidak dilindungi insuran.

LAMPIRAN XX

Borang Keizinan bagi Penerbitan Bahan yang berkaitan dengan Pesakit
(Halaman Tandatangan)

Tajuk Kajian: ***Peranan khusus kevirulenan Helicobacter pylori dan faktor genetik perumah dalam penyakit gastroduodenal***

Nama Penyelidik: Hussein Ali Osman

Untuk menyertai kajian ini, anda atau wakil sah anda mesti menandatangani mukasurat ini.

Dengan menandatangani mukasurat ini, saya memahami yang berikut:

- **Bahan yang akan diterbitkan tanpa dilampirkan dengan nama saya dan setiap percubaan yang akan dibuat untuk memastikan ketanpanamaan saya. Saya memahami, walaubagaimanapun, ketanpanamaan yang sempurna tidak dapat dijamin. Kemungkinan sesiapa yang menjaga saya di hospital atau saudara dapat mengenali saya.**
- **Bahan yang akan diterbitkan dalam penerbitan mingguan/bulanan/dwibulanan/suku tahunan/dwi tahunan merupakan satu penyebaran yang luas dan tersebar ke seluruh dunia. Kebanyakan penerbitan ini akan tersebar kepada doktor-doktor dan juga bukan doktor termasuk ahli sains dan ahli jurnal.**
- **Bahan tersebut juga akan dilampirkan pada laman web jurnal di seluruh dunia. Sesetengah laman web ini bebas dikunjungi oleh semua orang.**
- **Bahan tersebut juga akan digunakan sebagai penerbitan tempatan dan disampaikan oleh ramai doktor dan ahli sains di seluruh dunia.**
- **Bahan tersebut juga akan digunakan sebagai penerbitan buku oleh penerbit jurnal.**
- **Bahan tersebut tidak akan digunakan untuk pengiklanan ataupun bahan untuk membungkus.**

Saya juga memberi keizinan bahawa bahan tersebut boleh digunakan sebagai penerbitan lain yang diminta oleh penerbit dengan kriteria berikut:

- **Bahan tersebut tidak akan digunakan untuk pengiklanan atau bahan untuk membungkus.**
- **Bahan tersebut tidak akan digunakan di luar konteks – contohnya: Gambar tidak akan digunakan untuk menggambarkan sesuatu artikel yang tidak berkaitan dengan subjek dalam foto tersebut.**

Nama Pesakit **(Dicetak atau Ditaip)** Nama Singkatan **atau** No. Pesakit

No. Kad Pengenalan Pesakit **T/tangan Pesakit** Tarikh **(dd/MM/yy)**

Nama & Tandatangan **Individu yang Mengendalikan** Tarikh **(dd/MM/yy)**

Perbincangan Keizinan (Dicetak atau Ditaip)

Nota: **i) Semua subjek/pesakit yang mengambil bahagian dalam projek penyelidikan ini tidak dilindungi insuran.**