ASSOCIATION OF *HELICOBACTER PYLORI CAG*A AND SPECIFIC *VAC*A GENE POLYMORPHISMS WITH CLINICAL OUTCOME IN PATIENTS WITH DYSPEPSIA PRESENTING AT KENYATTA NATIONAL HOSPITAL, KENYA

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Association of *Helicobacter pylori cag*A and Specific *vac*A Gene Polymorphisms with Clinical Outcome in Patients with Dyspepsia Presenting at Kenyatta National Hospital, Kenya

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

Signature..... Date.....

Lawrence Guantai M'itonga

This Thesis has been submitted for examination with our approval as University supervisors.

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DEDICATION

I dedicate this work to my late father Fredrick M'itonga who left us when I was only two years old. This goes to my lovely wife Esther Guantai and my children, Yvonne Kanana, Yna Kathomi and Alpha Gitonga as well as my mum, Esther M'itonga and the entire extended family members.

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LIST OF ABBREVIATIONS AND ACRONYMS

***cag*A** Cytotoxin associated gene

CLO Campylobacter like organisms

DNA Deoxyribo-nucleic acid

ELISA Enzyme Linked Immunosorbent Assay

GERD Gastro-esophageal Reflux Diseases

GOO Gastric Outlet Obstruction

HpSA *Helicobacter pylori* Stool Antigen

IFN Interferon

IgG Immunoglobin G

IL Interleukin

ITROMID Institute of Tropical Medicine and Infectious Diseases

JKUAT Jomo Kenyatta University of Agriculture and Technology

KEMRI Kenya Medical Research Institute

KNH Kenyatta National Hospital

LPS Lipopolyssaccharide

MALT Mucosa Associated Lymphoid Tissue

MHC Major Histocompatability Complex

mRNA Messenger RNA

NSAIDS Non-steroidal anti-inflammatory Drugs

RUT Rapid Urease Test

TNF Tumour Necrosis Factor

UBT Urea Breath Test

***vac*A** Vacuolating Cytotoxin Gene A

ABSTRACT

Helicobacter pylori is a spiral, Gram-negative micro-aerophilic bacterium that chronically infects the gastric mucosa of more than half of all people worldwide, and is a major cause of gastritis and peptic ulcer (PU) disease, and a risk factor for gastric adenocarcinoma and mucosa-associated lymphoid tissue lymphoma. The differences in disease outcome may be the result of host factors, environmental factors and differences in the prevalence or expression of bacterial virulence factors. Cytotoxin-associated gene A (*cag*A) and some isoforms of vacuolating cytotoxin (*vac*A) gene have been associated with disease severity. The oncogenic potential of *cag*A is linked to its polymorphic EPIYA motifs pattern whose combinations differ geographically. The aim of this study was to establish the association of the specific virulence-associated bacterial genotypes with the clinical outcome of *Helicobacter pylori* infection. In this study, *H. pylori* strains in gastric biopsy specimens from dyspeptic patients were characterized and associated *cag*A, *vac*A gene polymorphisms and *cag*A EPIYA motifs pattern with the clinical outcome of infection. One hundred and twenty seven dyspeptic patients were enrolled into the study of whom 63.8% had gastritis, 13.4%, gastro oesophageal reflux disease (GERD), 11.8% non-ulcer dyspepsia (NUD), 9.4% peptic ulcer, 7.9%gastric cancer, and 0.01% other gastro duodenal diseases. *H. pylori* positivity was determined by histology and molecular diagnostic method directly from the gastric pathologies. The *H. pylori* DNA was detected in 62.99% of the one hundred and twenty seven dyspeptic patients. The prevalence of *cag*A gene was 48.75% among the *H. pylori*-positive patients. The presence of *cagA* was not significantly associated with the gastro duodenal diseases. The less virulent *vac*A alleles: m2, i2 and s2 were most occurring at 65%, 52% and 49%, respectively. The *vac*A allele m1, i1, and s1 were significantly associated with peptic ulcer, intestinal metaplasia and gastric cancer, all at $P < 0.05$.There was no EPIYA ABD detected in these strains. The most occurring EPIYA pattern was the ABC (56.41%) followed by ABCC at 43% and AB at 28.21%. The presumed virulent ABCCC pattern was rare (5.13%). Increase in the number of EPIYA C repeats was insignificantly associated with peptic ulcer ($p = 0.768$). However, samples with more than one C repeat

were significantly associated with gastric cancer (OR=6.577 95% CI 1.620-26.704, p=0.008). The low prevalence of these strains might contribute to the low incidence of gastric cancer in this country. The *cag*A-positive *H. pylori* infection was averagely prevalent in dyspeptic patients in Kenya and all the CagA protein EPIYA patterns were of Western type. The less toxigenic *vac*A isoforms s2, m2 and i2 were highly distributed. This study confirmed the potential of *vac*A s1, m1 and i1 to induce gastric cancer and peptic ulcer. Further, it can be concluded that determining the EPIYA motifs in CagA protein, rather than detecting *cag*A gene alone, would be a better marker for assessing the risk of serious gastric pathology. The possibility of the presence of unidentified strain or certain host or environmental factors that trigger *H. pylori*-induced cancer and peptic ulceration cannot be ruled out.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Helicobacter pylori (*H. pylori)* is estimated to infect the stomachs' of half the world's population and is associated with the development of gastro duodenal diseases, including peptic ulceration and gastric cancer (Ernst *et al.*, 2000). The differences in disease outcome may be the result of host factors, environmental factors and differences in the prevalence or expression of bacterial virulence factors (Covacci *et al.*, 1999). The study of *H. pylori* virulence factors in populations is important, as they contribute to disease risk. *H. pylori* strains express various toxins that enable the bacteria to cause host cell damage (Montecucco & Rappuoli, 2001). Included among these toxins is cytotoxin-associated gene A (*cag*A) and vacuolating cytotoxin (*vac*A) (Montecucco & Rappuoli, 2001). *H. pylori* strains possessing the *cag*A Pathogenecity Island (ca*g*A) are associated with the development of gastric cancer **(**Blaser *et al.*, 1995). The CagA protein is translocated into epithelial cells and becomes phosphorylated on tyrosine residues within EPIYA motifs, which may be repeated within the variable region of the protein (Higashi *et al.*, 2002). The cagA has a structural diversity in the EPIYA-repeat segment of which four types are described as A, B, C, and D (Higashi *et al.*, 2002; Naito *et al.*, 2006). On the basis of the EPIYA –repeat the cagA protein is classified into two types; the western and East Asian types. Both types have A and B however, they differ in that EPIYA C is specifically found in the Western type and EPIYA D in the East Asian type (Azuma *et al.*, 2004). EPIYA C is often duplicated (Higashi *et al.*, 2002; Naito *et al.*, 2006).

Phosphorylated CagA protein forms a physical complex with SHP-2 phosphate triggering abnormal cellular signals leading to deregulation of cell growth, cell to cell contact and cell migration, elongation of epithelial cells and increase of epithelial cell

turnover enhancing the risk of damaged cells acquiring precancerous genetic changes (Higashi *et al.*, 2002; Naito *et al.*, 2006). The EPIYA C and D are the main sites of phosphorylation of the CagA protein. EPIYA D and Multiple EPIYA C repeats were associated with increased SHP- Phosphate activity induced by CagA protein (Higashi *et al.*, 2002). An epidemiological evidence that there was a significant association between the development of cancer and infection with *H. pylori* strains carrying EPIYA ABD *cag*A genotype was presented in South Korea(Jang *et al.*, 2004); an association that had earlier been made elsewhere (Satomi *et al.*, 2006). Studies have also shown that strains with *cag*A with higher number of EPIYA C segment to be closely associated with gastric cancer than strains with *cag*A with one EPIYA C segment only (Yamaoka *et al.*, 1998; Hatakeyama *et al.*, 2009).

VacA toxin is produced and secreted by all *H. pylori* strains and was previously shown to have various modes of action (Cover & Blanke, 2005). Like *cag*A, *vac*A has been shown to contain a number of polymorphisms (McClain, *et al.*, 2001). Currently, three polymorphic regions of *vacA* have been identified: the signal (s), intermediate (i), and middle (m) regions (McClain, *et al.*, 2001). Each of these polymorphic regions has two main types that divide them further into type 1 and type 2 (McClain, *et al.*, 2001). The s region encodes the N-terminal signal sequence (McClain, *et al.*, 2001), and polymorphisms in the s region affect the anion channel-forming efficiency of the toxin; the s1 type has an increased ability to form membrane channels (McClain, *et al.*, 2001). Polymorphisms in the m region affect the cell tropism of the toxin; the m1 type of *vac*A shows toxicity toward a broader range of cells than the m2 type (Pagliaccia, *et al.*, 1998). The i region, located between the s and m regions, also displays two main polymorphisms (Rhead, *et al.*, 2007). The i1 type of *vac*A has stronger vacuolating activity than the i2 type. Individually, the s1, i1, and m1 types have been shown to be associated with more severe forms of *H. pylori* induced disease (Rhead, *et al.*, 2007; Basso, *et al.*, 2008).

1.2 Problem Statement

An intriguing aspect of *H. pylori* is an extraordinary diversity among strains that is seen in DNA fingerprinting and in tests of gene content and chromosomal gene order (van der Ende *et al.*, 1996). In particular, the DNA sequence motifs and disease association of two virulence genes, *vac*A and *cag*A, in strains from the United States and Europe differ from those predominating in southern coastal China and Japan, although less phylogenetic clustering is found in sequences of several housekeeping genes (Yamaoka *et al.*, 1999; Basso *et al.*, 2008).

The geographic distribution of distinct *H. pylori* genotypes remains largely unknown (Covacci *et al.*, 1999). The prevalence of virulent bacterial genotypes in certain regions may have important epidemiological consequences that are linked to the presence of certain specific genotypes and the severity of *H. pylori*-related diseases (Covacci *et al.*, 1999). The number and pattern of phosphorylation motifs seem to further stratify the risk associated with individual strains (Higashi *et al.*, 2002). It has been demonstrated that *H. pylori cag*A EPIYA motifs patterns have a significant geographic variability and closely follow patterns of historical human migrations (Azuma *et al.*, 2004).

Therefore, there is need to gain new insights into the population genetic structure of this important human pathogen and to learn if genotypes and *cag*A EPIYA motifs patterns implicated with the disease in the West as well as Eastern Asia are similarly associated with disease in Kenya.

1.3 Justification

Each human population has a characteristic distribution of *H. pylori* strains that typically infects members of that population (Bani-Hani, 2002). The differences in disease outcome may be the result of host factors, environmental factors and differences in the prevalence or expression of bacterial virulence factors (Bani-Hani, 2002). The complex relation between the genotype of *Helicobacter pylori* and its association with clinical

outcome is not well understood (van der Ende *et al.*, 1996). Studies in the West have showed that strains expressing certain virulence factors (*vac*As1, *vac*Am1, and *cag*A) are associated with duodenal ulcer disease. However, the *H. pylori* genotype is known to vary with geographic region (Bani-Hani, 2002). Strains possessing *cag*A with greater numbers of EPIYA motifs repeat have been more closely associated with gastric carcinogenesis (van Doorn *et al.*, 1999; Gomes *et al.*, 2008).

Most of the present understanding of *H. pylori* genome organization and bacterial traits are based on studies of strains from the West, and it is believed that further studies of *H. pylori* genotypes from different well-separated human populations will help increase our understanding of bacterium-host interactions in colonization and disease. Prevalence studies have indicated that *H. pylori* infection is extremely common in Kenya as in other developing countries. Available reports from Kenya have focused on the clinical patterns of gastro duodenal disease, detection of *H. pylori* infection and effectiveness (or ineffectiveness) of anti-*H. pylori* therapies in the local population, but there is limited information on the genotypes and *cag*A EPIYA motifs pattern of the Kenyan *H. pylori* strains.

In this context we have characterized *H. pylori* strains in gastric biopsy specimens from dyspeptic patients presenting at Kenyatta National Hospital in order to gain new insights into the population genetic structure of this important human pathogen and to learn if genotypes and *cag*A EPIYA motif patterns implicated with the disease in the West as well as Eastern Asia are similarly disease associated in Kenya.

1.4 Research Questions

1. What are the demographic characteristics and endoscopic findings of the patients presenting to the gastroenterology clinic in Nairobi with dyspepsia?
2. What is the distribution of the *cag*A and *vac*A gene polymorphisms of *Helicobacter pylori* in patients with various gastro duodenal diseases?

3. Is there any association between *cag*A and *vac*A gene polymorphisms with the clinical outcome of infection?
4. Is there any association between specific *cag*A EPIYA motifs pattern with the clinical outcome of the infection?

1.5 Hypothesis

Clinical outcome of *Helicobacter pylori* infection is not associated with *cag*A, specific *vac*A gene polymorphisms and *cag*A EPIYA motifs pattern.

1.6 Objectives

1.6.1 Main Objective

To establish the association of *Helicobacter pylori cag*A, specific *vac*A gene polymorphisms and *cag*A EPIYA motifs pattern with clinical outcome in patients with dyspepsia presenting at Kenyatta National Hospital, Kenya

1.6.2 Specific objectives

1. To describe demographic characteristics and endoscopic findings of the patients presenting to the gastroenterology clinic in Nairobi with dyspepsia.
2. To determine the distribution of the *cag*A and specific *vac*A gene polymorphisms of *Helicobacter pylori* in patients with various gastro duodenal diseases.
3. To determine the association between *cag*A and specific *vac*A gene polymorphisms with the clinical outcome of infection.
4. To determine the association between *cag*A EPIYA motifs pattern with the clinical outcome of the infection

CHAPTER TWO

LITERATURE REVIEW

2.1 *Helicobacter Pylori* and its Associated Diseases

H. pylori is a spiral shaped, Gram negative rod that colonizes human beings (Marshall *et al.,* 1984). In 1983, Warren and Marshall reported isolation of these spiral organisms from gastric mucosal biopsy specimen of patients with peptic ulcer disease and chronic active gastritis. These catalase positive organisms have 4-6 sheathed flagella attached to one pole, unlike the unsheathed flagella of *Campylobacter* (Thomas *et al*., 2004). The organisms are 0.5 – 1.0µm in diameter by 2 - 5 µm long. Though spiral they may be curved or straight. Under condition of environmental stress, *H. pylori* will convert from a spiral to a coccoid form (Marshall *et al.,* 1984). With its flagella and spiral shape, the bacterium drills into the mucus layer of the stomach and can either be found suspended in the gastric mucosa or attached to epithelial cells. The ecological niche of *H. pylori* is the human stomach where it establishes long term colonization of the gastric mucosa. The gastric antrum is the most favoured site, but any part of the stomach may be colonized (Blaser, 1997).

H. pylori is the most common chronic infection in human beings, affecting more than half of the world population (Graham & Graham, 1998). About 70% of those who are affected are asymptomatic (Thomas *et al*., 2004). The organism can cause acute gastritis, chronic active, chronic persistent and chronic atrophic gastritis. It is strongly associated with peptic ulcers (Ogutu *et al*., 1998; Karari *et al.,* 2000) and ensuing complications of bleeding and iron deficiency. It is also associated with mucosa – associated lymphoid tissue lymphoma, gastric adenocarcinoma, gastric cancer and possibly non ulcer dyspepsia (Parsonnet, 1998). A prevalence of 90% and 75% of *H. pylori* in patients with duodenal ulcer and gastric ulcers was reported, respectively (Kidd

et al., 1999). *H. pylori* is now classified as group 1 (or definite) carcinogen by the WHO International Agency for Research on Cancer (Michael *et al*., 2002).

2.2 Mode of Transmission

Three modes of transmission have been described. These include: oro-faecal, oro-oral and waterborne (Megraud, 1995). The infection is mainly acquired in childhood in both industrialized and developing countries, and persists throughout life unless treated (Malaty & Graham, 2004). Infection may also occur in adult life, which would explain the above average rates of infection in some occupational groups. In some studies, gastroenterologists have been shown to be significantly more likely to be infected than aged matched controls. This would raise the possibility of transmission from patient to doctor during endoscopies. Iatrogenic spread through contaminated endoscopies has been documented but can be prevented by proper cleaning of equipment (CDC, 2004).

2.3 Prevalence

The prevalence of infection varies both among countries and within different racial groups' resident within the same country. In both developed and developing countries, the highest rates of infection are associated with low socio-economic status, crowding, poor sanitation and unclean water supplies (Graham & Graham, 1998).

In general, in developing countries, >50% of children are infected by age of 10 years, the prevalence of infection rising to >80% in young adults (Misiewicz & Harris, 1995). In contrast in the majority of developed countries, children become infected at a rate less than 1% a year (Graham & Graham, 1998). Indeed, it is this significant difference in the rate of childhood acquisition of infection that is responsible for the differences in prevalence of *H pylori* infection observed between developed and developing countries. A study in Soweto, South Africa, found 46% of children at 1 year and 100% of children at 12 years to be infected with *H. pylori* (Ally & Segal, 1998).

The adult prevalence starts at 20 – 50% in the developed world and 50 – 80% in sub-Saharan Africa (Skalsky, 1995). A prevalence of 81 % of *H. pylori* infection in dyspeptic patients using histopathology and stool antigen test in Ethiopia was reported (Astrat, *et al.,* 2004). In Kenya, a prevalence of 81.7% in dyspeptic patients presenting to Kenyatta National Hospital was reported (Ogutu, *et al.,*1998), while a prevalence of 84.6% was found in HIV negative patients with upper gastrointestinal tract symptoms in the same hospital (Ali, *et al.,*2002).

2.4 Disease Patterns

H. pylori is found predominantly in human gastric mucosa. It lies adhered to the gastric epithelial cells, but is not locally invasive. Colonization often extends into the gastric glands, but the organisms do not invade the epithelial cells. Early exposure to *H. pylori* has been associated with widespread colonization of the stomach lining and a tendency to chronic gastritis. This may be patchy initially, involving superficial and glandular portions of gastric mucosa. It progresses to severe glandular destruction, with atrophy and metaplasia (Tsuji, *et al*., 2003). Metaplasia is an important predisposing factor for gastric cancer. Exposure to *H. pylori* at a later age has been associated with more localized stomach colonization, mainly the antrum. Destruction of antral D –cells in this region is associated with loss of somatostatin, which leads to increased release of gastrin. It was postulated that acute infection and / or inflammation of the antrum stimulated increased release of gastrin, a consequence of depletion of somatostatin (McColl, *et al*., 1998). This increased gastrin stimulates the parietal cells to secrete excess acid. The degree of increased acid released is dependent on the parietal cell mass. The resulting increased duodenal acid load leads to the development of gastric metaplasia and eventually ulceration. In patients who develop gastric ulcer and gastric cancer, infection and gastritis involves the acid-secreting body mucosa and is associated

with mucosal atrophy. Inflammation and atrophy of the acid secreting stomach mucosa results in hypochlorhydria or achlorhydria (McColl, *et al*., 1998).

There was a suggestion that most genetic factors that affect interleukin 1 – ß (a powerful inhibitor of gastric acid secretion) may determine why some individuals infected with *H pylori* develop gastric cancer and others do not (EL Omar, *et al*., 2000). Two related mechanisms by which *H pylori* could promote cancer are under investigation. One mechanism involves enhanced production of free radicals near *H. pylori* and an increased rate of host cell mutation. A second proposed mechanism, the "peri-genetic pathway", involves enhancement of the transformed host cell phenotype by means of alteration in cell proteins (Tsuji, *et al*., 2003). It has been proposed that *H. pylori* induce inflammation and high levels of TNF – Alpha and /or interleukin – 6. According to the proposed peri-genetic mechanism, inflammation – associated signaling molecules such as TNF- α can alter gastric epithelial cell adhesion and lead to the dispersion and migration of mutated epithelial cells without the need for additional mutations in tumour suppressor genes such as the genes that code for cell adhesion. The chronic T cell stimulation caused by *H .pylori* infection leads to release of inflammatory cytokine. These then stimulate B cell proliferation leading to MALT- Lymphoma.

It has been suggested that *H. pylori* in most people is harmless and may have potential benefits (Dent, 1998; EL Omar, *et al*., 2000). The organism may be protective against the development of gastro-oesophageal reflux and its adverse implications (Dent, 1998). This is also thought to be the case with hiatus hernia, Barrett's oesophagus and adenocarcinoma of oesophagus. The rationale for this protection is that *H. pylori* gastritis attending the corpus may produce gastritis severe enough to cause a major reduction of gastric acid secretion and a substantial elevation of gastric acid pH, compared to subjects who are not *H. pylori* infected (Dent, 1998). As a corollary, the decreasing rate of *H. pylori* in developed countries will result in a more effective preservation of acid secretion into old age, with a consequence greater prevalence of reflux oesophagitis and its attendant complication (Dent, 1998).

2.5 Colonization and Pathogenesis

The antrum of the stomach is a region of moderate acidity where *H. pylori* usually prefer to colonize first (Moran *et al.,* 1996; Hunt *et al.,* 1996). The bacterium uses its flagella and its spiral shape to drill through the mucus layer in the stomach (Hunt *et al.*, 1996). The *H. pylori* produces adhesions which bind to membrane associated lipids and carbohydrate. Electron microscopy reveals the fine filamentous strands of glycocalyx – like materials which extend between the organism and the gastric epithelial cell membrane (Hunt *et al.*, 1996; Moran *et al.,* 1996). Sometimes adhesion pedestal is present, which involves tight adhesion associated with local effacement of the microvillus (Michael *et al.,* 2002). Adhesins have been identified based on the characterization of bacterial proteins or identification of cellular receptors (Hunt *et al.*, 1996; Moran *et al.,* 1996). There has been evidence of tissue tropism in *H. pylori* colonization (Moran *et al.,* 1996). *Helicobacter pylori* produce copious amounts of cell surface - associated urease as a catalytically active heterodimer (Hunt *et al.*, 1996). This urease is secreted and elevates the local intragastric pH by catalyzing urea breakdown into ammonia and bicarbonate (Hunt *et al.*, 1996). The ammonia forms a cloud around the bacterium, shielding it from the gastric acid. Ammonia also reacts with water to form ammonium ions that neutralize the gastric acid. These properties contribute to the persistence of *H. pylori* infection in human stomach (Hunt *et al.*, 1996; Moran *et al.,* 1996). Its colonization of the duodenum is associated with sites of gastric metaplasia (Hunt *et al.*, 1996; Moran *et al.,* 1996; Labigne & Hilder, 1996).

Ammonia production from urease activity is toxic to mammalian cells (Labigne & Hilder, 1996). Epithelial cells undergo vacuolation because of urease activity (Labigne & Hilder, 1996). In addition, urease and other products of *H. pylori*, including proteases, catalase and phospholipase A_2 and C cause weakening of mucous bicarbonate layer of the gastrointestinal tract and damage to surface epithelial (Hunt *et al.*, 1996; Labigne & Hilder, 1996: Moran *et al.*, 1996). Lipopolysaccharide (LPS) seems to inhibit glycosylation of mucus leading to a significant change in the macromolecular structure

of mucin from high molecular weight to low molecular weight form (Moran *et al.*, 1996). Lipopolysaccharide may interfere with protective function of mucus layer and make epithelial cells at the surface vulnerable to acid (Moran *et al.*, 1996). Lipopolysaccharide from some strains of *H. pylori* stimulates secretion of pepsinogen, hence high levels of pepsinogen in ulcer patients. Lipid A component of LPS in *H. pylori* has unusual fatty acid substitution and is under phosphorylated compared to LPS in other Gram-negative bacteria. These modifications are known to reduce immunological activity (antigenicity) of LPS. This allows *H. pylori* to persist in its host as compared to a more aggressive pathogen (Moran *et al.*, 1996).

A vacuolating cytotoxin which induces cytoplasmic vacuolation in eukaryotic cells was first described by Leunk *et al.* (1988). Infection with toxin producing strains is more common in patients with peptic ulcer disease (Leunk *et al.*, 1988; Crover & Blaser, 1999). The biological activity of *vac*A protein is increased by exposure to acid (Censini *et al.*, 1996). The acid activated *vac*A is currently considered to be an important factor of increased mucosal damage in *H. pylori* infection (Censini *et al.*, 1996; Crover & Blaser, 1999). Cytotoxin – associated gene (*cag*A) positive strains have been consistently associated with increased levels of inflammation (Crover & Blaser, 1999).

Cytotoxin – associated gene A is part of a large pathogenicity island, the *cag* PAI, which encodes a type IV secretion system (Crover & Blaser, 1999). This system permits the transfer of bacterial proteins into the host cell, resulting in the transcription of pro-inflammatory cytokines such as IL –8, a molecule known to be responsible for recruitment of neutrophils (Crabtree, 1999). *In vivo*, infection with *cag*A positive strains results in increased mucosal immune responses and more intense gastritis (Yamaoka *et al.*, 1996).

2.6 Host Immune Responses

H. pylori survive within the stomach despite a powerful host immune response, demonstrated by high ant- *H. pylori* antibody titters. This indicates that the organism is capable of evading the host's immune response. *H. pylori* could either be able to down regulate the immune system or impair the ability of phagocytes to kill the bacteria. The fact that the organism is extra cellular also makes it less accessible to attack by the inflammatory cells (Shimoyama, *et al.*, 2000).

H. pylori can bind to class II major histocompatibility-complex (MHC) molecules on the surface of gastric epithelial cells, inducing their apoptosis (Ye G, *et al.*, 1997,) *H. pylori* urease and porins may contribute to extravasation and chemotaxis of neutrophils (Harris, *et al.*, 1996; Tufano, *et al.*, 1994). Further changes in epithelial cells depend on the translocation of cagA into gastric epithelial cells. Inflammatory cytokines are increased in patients infected with *cag*A positive phenotype. As described earlier cag A is not the direct inducer of IL –8 but cag A positive strains are associated with increased gastric IL –8 mRNA expression and IL –8 protein (Yamaoka, *et al*; 1996), a potent neutrophil chemokine. The inflammatory response to *H. pylori* consists of the recruitment of neutrophils, followed by T and B – lymphocytes, plasma cells and macrophages, with associated epithelial damage (Crabtree, 1996). There is increased release of pro-inflammatory IFN-γ, with a marked reduction in anti-inflammatory cytokines, IL-4, IL-5 and IL-10. However, a number of studies have reported IL –10 to be present in the gastric mucosa of subjects with *H. pylori*-related active gastritis (D' Elios, *et al.*, 1997). The above scenarios are expected with increased activity of Th1 Lymphocytes and often in response to intracellular organisms. *H. pylori* is known to be an extracellular organism, but it stimulates intracellular processes via the *cag*A protein (Crabtree, 1996; Yamaoka, *et al.*, 1996).

H. pylori infection induces vigorous systemic and mucosal humoral response. Predominantly, the IgG 1 (suggestive of Th2 response) was found in Sowetans and IgG 2 sub class (suggestive of aTh1 response) in Australian and German population (Mitchell, *et al.,* 2000). Almost all patients with chronic gastritis have a specific response to *H. pylori* by the gastric mucosal 1gG A. IgG plasma cells increased with gastritis. Almost all infected patients have IgG antibodies in serum (Crabtree, 1996).

Antibody production does not necessarily lead to eradication of the infection but may contribute to tissue damage. Some *H. pylori* infected patients have an autoantibody response directed against the H^+ / K^+- ATPase of gastric epithelial cells that correlates with increased atrophic gastritis (Negrini, *et al.,* 1996). Complement activation may also be another factor which contributes to neutrophilic response in gastric mucosa. The activated complement may have a role in neutrophil chemotaxis and epithelial damage (Berstad, *et al.*, 1997).

2.7 Treatment Recommendations

The current recommendations for eradication of *H. pylori* have been as a result of a consensus reached by leading gastroenterologists, for example National Institute of Health, USA (NIH Publication, 2004), the Maastricht consensus, Europe (Malfertheiner, *et al.,* 2002) and many others. The European consensus suggested that patients with gastritis with severe intestinal metaplasia or gastric atrophy and those who had early gastric cancer resected should also be screened for *H. pylori* followed by eradication treatment. Eradication of *H. pylori* in peptic ulcer disease and MALT Lymphoma has been associated with clinical resolution / regression of disease. Recurrence rates of duodenal ulcer at 1 year fall from 67% to 6% with eradication as opposed to anti-secretory treatment. These figures stand at 59% to 4% with gastric ulcers. The major complications of peptic ulcer disease are gastrointestinal bleeding, perforation, and gastric outlet obstruction. Treatment of *H. pylori* infection greatly reduced the incidence of these complications (Malfertheiner, *et al.,* 2002).

In the long-term, *H. pylori* eradication can reduce the cost of ulcer treatment by approximately ten fold. *H. pylori* eradication is also strongly recommended in *H. pylori* positive patients with low grade MALT Lymphoma, although subsequent lifelong surveillance is needed. Those with high grade MALT Lymphoma should undergo *H. pylori* eradication as first line treatment (Fischbach, *et al.*, 1999; Ruskone, *et al.*, 2001). Atrophic changes in the gastric mucosa are associated with an increased risk of possible progression to gastric cancer (Valle, *et al.*, 1996) and therefore this condition requires intervention by the eradication of *H. pylori* (Sung, *et al.*, 2000), although no proof that progression to neoplasia occurs.

Though there is no recommendation to screen asymptomatic individuals, Maastricht 2 – 2000 report, (Malfertheiner, *et al.*, 2002), strongly recommended *H. pylori* eradication in infected individuals who are first degree relatives of gastric cancer patients and in *H. pylori* positive patients following consultation with their physician. Although it has not been proven that the eradication of *H. pylori* will result in protection against gastric cancer in first degree relatives of gastric cancer patients, this group is at significantly higher risk than the general population (Tepes, *et al.*, 1999; EL – Omar, *et al.*, 2000).

The report recommended the screening for all dyspeptic patients requiring long –term non-steroidal anti-inflammatory drugs (NSAIDs). *H. pylori* eradication is advisable if NSAIDs therapy is planned in order to eliminate the infection as a confounding explanation of subsequent peptic ulcer and dyspeptic symptoms (Malfertheiner, *et al.*, 2002). Additionally, *H. pylori* eradication is an advisable option in infected patients with functional dyspepsia (NIH Publication, 2004).

2.8 Treatment Regimens

H. pylori peptic ulcers are treated with drugs that kill the bacteria reduce stomach acid and protect the stomach lining. Antibiotics kill the bacteria whilst acid suppressing

drugs such as H_2 blockers and proton pump inhibitors reduce acid secretion in the stomach (NIH Publication, 2004).

H_2 blockers work by blocking histamine, which stimulates acid secretion. They help reduce ulcer pain after a few weeks. Proton pump inhibitors suppress acid production by halting the mechanism that pumps the acid into the stomach. Bismuth subsalicylate, a component of Pepto-Bismol, is used to protect the stomach lining from acid. It also kills the bacteria (NIH Publication, 2004).

The antibiotic used include Tetracycline, Amoxicillin, Clarithromycin, Metronidazole (or other Nitroimidazole), Furazolidone and a semi synthetic Ansamycin, the Rifabutin. During treatment, one should take into account factors such as cost, effectiveness, compliance, side effects and resistance (NIH Publication, 2004). The Maastricht 2 – 2000 consensus report recommended that a first – line therapy should be with triple therapy using a proton pump inhibitor or ranitidine bismuth citrate, combined with Clarithromycin and Amoxicillin or Metronidazole. Subsequent second –line therapy should use quadruple therapy with a proton pump inhibitor, bismuth, Metronidazole and Tetracycline and where Bismuth is not available second line therapy with a proton pump inhibitor triple therapy (Unge, *et al.*,1998; Lind, *et al.*, 1999). If second line therapy fails in primary care, patients should be referred to enable specialist assessment, including antibiotic susceptibility testing.

2.9 Diagnosis of *Helicobacter Pylori* Infection

Infection with *H. pylori* can be diagnosed either by invasive techniques requiring endoscopy and biopsy such as histological examination, rapid urease test, culture, polymerase chain reaction (PCR) or by one of the several non-invasive testing methods such as serologic tests, urea breath tests, stool antigen tests and stool PCR tests (NIH Publication, 2004).

2.9.1 Histology

Organism can be seen on Haematoxylin and Eosin – stained biopsies as well as with special stain's including modified Giemsa, Steiners, cold Ziehl Neelsen stain, Warthin Starry Silver and Acridine orange. The advantage of histology is that besides detection of *H. pylori*, other abnormalities can be detected, for example malignancy, dysplasia or lymphoma (NIH Publication, 2004).

In addition to the type of stain used, uneven distribution of the organism through the gastric mucosa influences histological investigation. Antral biopsies are generally recommended. Sensitivity and specificity of histological testing are approximately 95% and 98% respectively (Lin, *et al.*, 1992).

2.9.2 Rapid Urease Test.

This involves rapid urease testing of gastric biopsy material. The *H. pylori* urease catalyzes the hydrolysis of urea into ammonia and bicarbonate. The net effect of ammonia production is increased local pH. Biopsy samples are placed on an agar gel or paper strip containing a pH indicator. If the *H. pylori* organisms are present in sufficient numbers in the antral biopsy sample, a colour change will occur as a result of urea breakdown and ammonia production by *H. pylori* urease. Commercial rapid urease tests include the Agar gel – based tests (CLO test, Delta West) or paper strip test (e.g. Pylori Tek, Horizons International). CLO test uses phenol red as the sensitive dye (American Society of Clinical pathologists, 2003)

This test can give a result in 1 –2 hours. One may need to delay the reading up to 24 hours so as to reduce the chances of a false negative result in the case of low *H. pylori* load. A sensitivity and specificity of 89% and 98.1% was reported, respectively (Leen-Jan van, *et al.*, 2000). Rapid Urease tests with faster reactions times are being evaluated (Franco, *et al.*, 2005). ESOKIT Hp test (Cambridge life sciences, UK) contains urea,

phenol red and buffer salts in tablet form. Sensitivity of detection depends on organisms load in the mucosal biopsy specimen (Franco, *et al.,* 2005).

2.9.3 Culture

Culture of *H. pylori* from gastric mucosal biopsies is considered the gold standard for diagnosis of infection. Since *H. pylori* are micro-aerophilic organisms, they grow in environments with lowered oxygen tension (5-10%), carbon dioxide raised to 5 – 10%, hydrogen environment (5-10%) and maintained at 35-37^{0}C for at least 7 days. Culture of organisms is particularly useful in identifying the pathogenic strains of *H. pylori* including cytotoxin production. In addition, antimicrobial sensitivity testing can be undertaken (John, *et al.,* 1997)

The sensitivity and specificity of this test are approximately 90% and 100% respectively (Leen, *et al.,* 2000). The sensitivity of this test is diminished if the numbers of organisms is small, culture techniques are inadequate, or if the patient has recently taken antimicrobial therapy. Because of cost and expertise required for culture of *H. pylori*, routine assessment is not currently recommended (John, 1997).

2.9.4 Molecular testing methods

Molecular methods such as PCR and southern blot hybridization have the capability to sensitively and accurately determine both the presence of infection and genotype of bacteria (Franco, *et al.,* 2005). Jeremy, *et al.,* (2000) documented the ability to detect *H. pylori* and perform molecular resistance testing by PCR –based assays with gastric biopsy. More so they claimed that MRT (molecular resistance testing) might be performed using the archived biopsy so that in case of therapy failure, the regimen may be modified based on genotyping results (Jeremy, *et al.,* 2000).

Faecal DNA purification involving gene capture coupled with PCR is an effective tool for obtaining *H. pylori* DNA from the faeces (Mackay, *et al.*, 2003). A major problem in the application of molecular techniques to gut micro-organisms is that human faeces containing inhibitory compounds of bacteria and / or plant origin such as complex polysaccharides that inhibit further processing of purified DNA (Mackay, *et al.*, 2003).

2.9.5 Serology tests

Serologic testing detects the presence of specific IgA, IgG, or IgM-class serum antibodies to *H. pylori* in a patient's serum. These antibodies are present in serum about 21 days after infection and can remain present long after the organism is eradicated. They can be assessed quantitatively using ELISA and Latex agglutination techniques or qualitatively using office based kits. Dozens of different serologic tests are commercially available (Vakil & Fendrick, 2005). Advantages of the serologic tests are their wide availability, their rapid results, and the fact that they require no specialized equipment or techniques and their cost relative to active tests.

The sensitivity of these tests is quoted as 80 – 95% and specificity 80 – 95% (Schembri, *et al.*, 1993). A specificity of 79% was reported (Viara & Vakil, 2001). The major disadvantage of serologic tests is that, they cannot distinguish between active infection and past infection with *H. pylori*, hence not clinically useful for monitoring patients post – *H. pylori* eradication therapy (John, 1997).

2.9.6 Urea Breath Tests

Urea breath test (UBT) identifies active *H. pylori* infection through the organism urease production. The patient ingests urea labelled with either the non-radioactive isotope carbon 13 (Breath Tek UBT for H. Pylori, Meretek Diagnostics, Inc, Lafayette, CO) or the radioactive isotope carbon 14(^{14}C), (PY test, Kimberley – Clark Corp, Draper, UT) (Mackay, *et al.*, 2003). If *H. pylori* are present in the stomach, hydrolysis occurs and labeled carbon dioxide is produced which is detectable within a few minutes in the

patient's breath. The labeled urea is typically given to the patient with a test meal to delay gastric emptying and increase contact time with the mucosa. After urea ingestion breath samples are collected for up to 20 minutes by exhaling into a carbon dioxide trapping agent. Though the amount of radiation in the ^{14}C urea breath test is less than daily background exposure (Goddard & Logan, 1997), the ^{13}C test is preferred in children and pregnant women (Graham & Kelin, 2000).The urea breath test detects active *H. pylori* infection and is highly accurate with a weighted mean sensitivity and specificity from published trials of 94.7% and 95.7%, respectively (Vaira & Vakil, 2001).

The limitation of UBT is that, ^{13}C UBT requires special equipment and is expensive and ^{14}C UBT requires a radioisotope and cannot be used for children and pregnant women. As a result, UBT is not widely available (Yi – Hui, *et al.*, 2004).

2.9.7 Stool Antigen Tests

There is evidence that infected individuals excrete *H. pylori* in faeces (Mapstone, *et al.*, 1993). The Premier Platinum HpSA (Meridian Diagnostics, Europe) an enzyme immunoassay for the detection of *H. pylori* antigens in human stool, cleared by the Food and Drug Administration and CE marked is now widely used in many countries, as a valuable tool for the *H. pylori* patients' management. The test utilizes polyclonal anti-*H. pylori* capture antibody adsorbed to micro wells. Diluted patient samples and peroxidase conjugated polyclonal antibody are added to the wells and incubated for one hour at room temperature. A wash is performed to remove unbound material. The substrate is added and incubated for ten minutes at room temperature. Colour develops on the presence of bound enzyme. Stop solution is added and results are interpreted visually or spectrophotometrically. This ELISA stool antigen test detects active *H. pylori* infection and is highly accurate, with a weighted mean sensitivity and specificity from published trials of 93.1% and 92.8% respectively (Vaira & Vakil,2001) rates that are virtually the same as those for the urea breath test.

Other kits such as the HpStAR (Dako, Glostrup, Denmark or Femtolab, Connex, Martirisried, Germany) use monoclonal antibodies because obtaining polyclonal antibodies of constant quality is difficult (Franco, *et al*; 2005). Many stool antigen tests have been developed, such as the HePy stool test (Biolife Italian, Italy) and *H. pylori* antigen EIA (International Ltd, Hong Kong). Immunochromatographic tests with monoclonal antibodies have been developed (Mackay, *et al.,* 2003).

2.10 Genetic Diversity of *Helicobacter Pylori* and Association with Gastro-Duodenal Pathologies

The genetic diversity of *Helicobacter pylori* is analyzed at two different levels: the genomic variation between strains originating from different individuals, and the variation in bacterial populations within an individual host (Marais *et al.,* 1999). The cytotoxin-associated-gene (*cag*) pathogenecity island (PAI) is an approximately 40-kb cluster of genes in the *H. pylori* chromosome and is divided into two regions, *cag*I and *cag*II (Censini *et al*., 1996). There are at least 14 and 16 open reading frames (ORFs) in *cag*I and *cag*II, respectively (Censini *et al*., 1996; Akopyants *et al.,* 1998). Some of the ORFs in the *cag* PAI are believed to encode proteins which have similarities to other bacterial secretion systems, such as the *Bordetella pertussis* toxin secretion system (Censini *et al*., 1996; Akopyants *et al.,* 1998).

The *cag* PAI is one of the major virulence factors of *H. pylori* (Parsonnet *et al*., 1997). Studies of the *cagA* gene, located in the most downstream portion of the *cag* PAI have indicated that the *cag*A is associated with peptic ulcer disease, gastric cancer, and mucosa-associated lymphoid tissue lymphoma in the stomach. The *cag*A antibodies were more frequently detected in *H. pylori*- infected patients with gastric cancer than in those without gastric cancer (odds ratio, 1.9) (Blaser *et al.,* 1998) Further, subjects infected with *H. pylori* who had *cag*A antibodies were more likely to develop gastric cancer as compared with uninfected subjects (odds ratio, 5.8), while *H. pylori*-infected

subjects without *cag*A antibodies were at only slightly and not significantly increased risk for cancer (odds ratio, 2.2) (Parsonnet *et al.*, 1997)

The proteins encoded by *cag* PAI genes are thought to function as a secretion system for export of bacterial factors involved in host epithelial activation (Blaser, 2004). *cag*A belongs to a *cag* PAI (Pathogenicity Island) that codes a type IV secretion system (T4SS) associated with increased secretion of IL-8, a very strong proinflammatory chemokine that participates in the gastritis induced by *H. pylori* infection (Blaser, 2004). The T4SS is also responsible for the entrance of CagA protein into the gastric epithelial cells where it is phosphorylated on the tyrosine residue within the phosphorylation motifs in the carboxiterminal variable region of the protein (Blaser, 2004). These motifs are defined as EPIYA (Glu-Pro-Ile-Tyr-Ala) A, B, C and D according to different flanking amino acids. CagA protein nearly always possesses EPIYA A and B segments that are followed by none, one, two or three C segments, in strains circulating in the Western countries, or a D segment, in East Asian countries. The EPIYA C and D are the main sites for phosphorylation of CagA (Asahi *et al.*, 2000).

Phosphorylated CagA forms a physical complex with SHP-2 phosphatase and triggers abnormal cellular signals leading to deregulation of cell growth, cell to cell contact and cell migration, elongation of epithelial cells and increase of epithelial cell turnover, which enhance the risk of damaged cells to acquire precancerous genetic changes (Higashi *et al.*, 2002; Naito *et al.*, 2006). Carrying the type D EPIYA or multiple C repeats is associated with increased SHP-2 phosphatase activity induced by CagA protein (Higashi *et al.*, 2002; Naito *et al.*, 2006), which raises the possibility that infection by *cag*A strains possessing higher number EPIYA C segments predisposes to precancerous lesions and gastric cancer. In fact, this hypothesis has been tested in Eastern countries, but the study results are discordant. There was increased proportion of EPIYA D strains among patients with atrophic gastritis and gastric cancer (Azuma *et al.* 2004), but other authors have been unable to reproduce these results (Zhu *et al.*, 2005; Choi *et al.*, 2007). Similarly, in Western populations, significant association between

gastric cancer and increased number of EPIYA C motifs could be demonstrated in two studies (Yamaoka *et al.*, 1999; Basso *et al.*, 2008), maybe either by the small number of included patients in the other studies (Argent *et al.* 2004; Acosta *et al.*, 2010; Sicinshi *et al.*, 2010), or by regional/ethnics differences as already demonstrated for other *H. pylori* virulence markers (van Doorn *et al.*, 1999; Gomes *et al.*, 2008).

Further, discrepancies have been demonstrated in studies evaluating the number of EPIYA C motifs and duodenal ulcer (Basso *et al.*, 2008; Salih *et al.*, 2010), which deserves in deep investigations because duodenal ulcer and gastric cancer are mutually exclusive *H. pylori*-associated diseases. Infection by *H. pylori cag*A-positive strains harboring multiple EPIYA C repeats is associated with gastric precancerous lesions and gastric cancer, but not with duodenal ulcer in an ethnically diverse, admixed, Western population (Higashi *et al.*, 2002; Naito *et al.*, 2006). Although infection by *H. pylori cag*A-positive strains is a risk factor for the mutually exclusive diseases, gastric cancer and duodenal ulcer, *cag*A strains possessing higher number of EPIYA C segments were associated with gastric cancer, but not with duodenal ulcer (Batista *et al.*, 2011)

The VacA toxin that is produced and secreted by all *H. pylori* strains was previously shown to have various modes of action (Cover & Blanke, 2005). It has been shown to contain a number of polymorphisms (McClain, *et al.*, 2001). Three polymorphic regions of *vacA* have been identified: the signal (s), intermediate (i), and middle (m) regions. Each of these polymorphic regions has two main types that divide them further into type 1 and type 2. The s region encodes the N-terminal signal sequence (McClain, *et al.*, 2001). The polymorphisms in the s region affect the anion channel-forming efficiency of the toxin (McClain, *et al.*, 2001). The s1 type has an increased ability to form membrane channels (McClain, *et al.*, 2001). Polymorphisms in the m region affect the cell tropism of the toxin. The m1 type of *vac*A shows toxicity toward a broader range of cells than the m2 type (Pagliaccia, *et al.*, 1998). The i region, located between the s and m regions, also displays two main polymorphisms (Rhead, *et al.*, 207). The i1 type of *vac*A has stronger vacuolating activity than the i2 type. Individually, the s1, i1, and m1 types have

been shown to be associated with more severe forms of *H. pylori* induced disease (Rhead, *et al.*, 2007; Basso, *et al.*, 2008).

The VacA protein plays a role in the pathogenesis of both peptic ulceration and gastric cancer (Artherton *et al.*, 1995; Marchetti *et al.*, 1995; Ogura *et al.*, 2000; Wada *et al.*, 2004). Although all strains carry a functional *vac*A gene, there is considerable variation in vacuolating activities among strains due to the sequence heterogeneity within the *vac*Agene (Artherton *et al.*, 1995; van Doorn *et al.*, 1998). Vacuolating activity is high in s1/m1 genotypes, intermediate in s1/m2 genotypes, and absent in s2/m2 genotypes (Artherton *et al.*, 1995). In line with this, *vac*As1/m1 genotypes are more frequently associated with peptic ulceration and gastric carcinoma (Artherton *et al.*, 1995). Even within one specific patient-strain combination, the VacA toxin expression levels differ over time due to the rapid evolution of the bacterium, which seems to be constantly adapting its genetic makeup to facilitate persistent infection (Falush *et al.*, 2001; Kuipers *et al.*, 2000; Aviles-Jimenez *et al.*, 2004). This microevolution also results in altered toxicity, and the constantly changing toxicity may (in part) explain the constant growing and shrinking of ulcers (Aviles-Jimenez *et al.*, 2004). VacA toxin forms pores in epithelial cell membranes, thus inducing the release of urea and anions from the host cells. It also increases transcellular permeability, leading to the release of nutrients and cations (Montecucco & de Benard, 2003). Interestingly, a significant part of the secreted toxin is not released into the environment but remains associated with the outer membrane of *H. pylori* (Ilver *et al.*, 2004; Fitchen *et al.*, 2005).

Upon bacterial contact with host cells, these toxin clusters are transferred to the host cell surface and exert their toxic action (Ilver *et al.*, 2004). This contact-dependent direct delivery mechanism suggests the involvement of specific receptors that mediate the bacterium-cell contact (Fitchen *et al.*, 2005). However, such a receptor has not yet been identified. There is a strong correlation between toxin activity and the pathogenicity of *H. pylori*, with the s1/m1 type of *vac*A being the most virulent in Western populations (Artherton *et al.*, 1995; Artherton *et al.*, 1997; Gunn *et al.*, 1998; van Doorn *et al.*, 1998; Wang *et al.*, 1998; Keates *et al.*, 1999). In contrast, such an association was not observed when Asian subjects were studied (Yamaoka *et al.*, 1997).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Design

This was a cross-sectional descriptive study.

3.2 Study Population

The study population included patients with past and present history of dyspepsia and had been referred for endoscopy at Kenyatta National Hospital (KNH). They included patients of either gender or any age group as long as they met the stipulated inclusion criteria.

3.3 Study Site

Kenyatta National Hospital (KNH), a national referral hospital in Kenya. It is a teaching hospital, and provides a medical research environment. It has fifty (50) wards, twenty two (22) out-patient clinics, twenty four (24) theatres (16 specialised) and Accident & Emergency Department. Out of the total bed capacity of one thousand eight hundred (1800) beds, two hundred and nine (209) beds are for the Private Wing. The hospital offers a wide range of diagnostic services such as laboratories, radiology/imaging and endoscopy among other specialised services. The hospital hosts in its wards between two thousand five hundred (2500) and three thousand (3000) patients in a day. On average the hospital caters for over eighty thousand (80,000) in-patients and over five hundred thousand (500,000) out-patients annually.

3.4 Inclusion Criteria

a) All patients presenting with past or present record of dyspepsia and had been referred for endoscopy for assessment of organic disease and had not been on any antibiotics or acid suppressive drugs within 2 weeks prior to presentation.

b) All patients with past records of dyspepsia and presented with a complication relating to disease states known to cause dyspepsia (Appendix 4)
c) Written informed consent (Appendix 1&2) by the patient or parent /guardian if the patient was less than 18 years old.

3.5 Exclusion Criteria

a) All patients who present without past or present record of dyspepsia though they had been referred for endoscopy
b) All patients who presented with past or present record of dyspepsia but had been on antibiotics or acid suppressive drugs within 2 weeks prior to presentation.
c) All patients who failed to provide a written informed consent (Appendix 1&2).

3.6 Sampling

Consecutive patients with past and present history of dyspepsia and who had been referred for endoscopy for assessment of organic disease at the gastroenterology clinic of the Kenyatta National Hospital were enrolled into the study.

3.7 Sample Size

Assuming 67.5% prevalence of *H. pylori* infection in dyspeptic patients (Kimang'a *et al.*, 2010) the target sample size was 125 dyspeptic patients, calculated using the formula (Chadha, 2006):

$$\frac{N = Z^2_{1-\alpha/2}(1-P)}{d^2}$$

Where n = sample size

Z = the standard normal deviate

P = estimated prevalence of the characteristic

d = degree of precision or accuracy, 0.1; (10%)

α = significance level

The confidence level is 95%

Z (1 – α/2) is the corresponding value to the 95% confidence level.

Therefore

$$N = \frac{1.96^2 (1- 0.675)}{0.1^2}$$

=125, this was the minimum sample size required.

3.8 Laboratory Methods

3.8.1 Demographic Data Collection and Endoscopy

After fulfilling the inclusion criteria and obtaining a written consent the patients provided the demographic data that included age and gender before undergoing routine endoscopy. Patients for Esophageal Gastro Duodenoscopy (EGD) underwent a detailed history and physical examination. Each patient was made to lie laterally. A mouth piece was introduced into the mouth. Esophageal Gastro Duodenoscopy was performed under conscious sedation with intravenous midazolam using a video-gastroscope. A thin, lighted tube with a camera on the end was eased carefully into the mouth and down the throat to the stomach and duodenum. The lining of the oesophagus, stomach and duodenum was observed. Antral and corpus biopsies, about 2-3 mm in length were taken

on an area of a normal looking tissue rather than an area affected by erosions or ulceration, because *H. pylori* could be smaller in numbers if epithelium was eroded or mucous layer denuded. Two biopsies per site were obtained for routine histology and molecular testing (DNA extraction). The biopsy specimens for molecular analysis were put in a2ml DESS (DMSO/ EDTA/ NaCl) solution and stored at $^{-}80^{0}$C at the Centre for Microbiology Research (CMR), KEMRI until use, while samples for histological analysis were immediately fixed in buffered formalin and processed at the Kenyatta National Hospital histopathology laboratory.

3.8.2 Histology and Giemsa Staining

About 2-3mm-biopsy specimens were fixed in 4% formalin overnight by a specialist. The tissues were then processed in a tissue processor. Ultramicrotomy was done in a microtome to produce 4-6 µm sections at the Kenyatta National Hospital histopathology laboratory. The sections were then deparaffinized and hydrated. They were then stained in fleshly prepared Giemsa working solution for 30 min – 1 h; dehydrated in 3 changes of absolute alcohol and cleared in xylene for 3 changes. They were then mounted with resinous medium and a cover slip placed. The slides were examined microscopically by using x400 and at least five high power fields examined.

3.8.3 Histopathology

The microscopic examination of tissue in order to study the manifestations of disease was carried out and scored as indicated in the sections below.

3.8.3.1 Assessment of Gastritis

A histopathological classification of gastritis was used using updated Sydney system (Dixon, *et al* 1996) which had a scale of 0 – 3 for scoring the features of chronic gastritis, corresponding to none, mild, moderate or severe respectively (Appendix 5).

3.8.3.2 Degree of Inflammatory Activity

This was scored according to the density of neutrophils in gastric mucosa; 0, none; 1, one or two crypt involved per biopsy; 2, 25 – 50% crypts involved per biopsy; 3, more than 50% or all crypts involved per biopsy.

3.8.3.3 Superficial Epithelial Damage

This was reported as none; slight or mild degeneration in the top of epithelial cells; moderate degeneration with disorientation of the epithelial lining indistinct cell border at the surface of the epithelium; flattened epithelial cell with severe degeneration and enlarged nuclei or flattened to erosive epithelium of the entire surface.

3.8.3.4 Degree of Chronic Inflammatory Infiltrate in the Gastric Mucosa (Lymphocytes, Plasma Cells)

This was reported as none; scattered chronic inflammatory cells or nearly to entire mucosa containing a dense chronic inflammatory cell infiltrate.

3.8.3.5 Degree of Intestinal Metaplasia

This was graded according to the amount of glandular tissue replaced by intestinal – type epithelium as: none; one to 4 crypts replaced by intestinal – type epithelium; moderate foci replaced or; nearly to entire area of gastric epithelium replaced by intestinal metaplasia.

3.8.3.6 Atrophy

This was defined as the loss of specialized gastric glandular tissue, partly characterized by replacement of intestinal metaplasia. The presence of intestinal metaplasia can be used as an indicator of the severity and extent of atrophy in antrum, but atrophy might occur without accompanying metaplasia and was graded as: none of gastric glands lost;

few and small areas in which gastric glands have disappeared or been replaced by intestinal –type epithelium; 25 – 50% of gastric stands or lost or replaced by intestinal – type epithelium or 50% or more of gastric glands lost or replaced by intestinal – type epithelium.

3.9 DNA Extraction

The samples were removed from DESS (DMSO/ EDTA/ NaCl) solution and soaked in 2 mL TE (10 mM Tris, 1 mM EDTA) for 2 h. The biopsies were then collected in microcentrifuge tubes containing 120 *u*l of sterile phosphate buffered saline and vortexed vigorously for 2 min. The tubes were then boiled in a water bath at 95 degrees centigrade for 15 min, cooled in ice, and centrifuged at 13,000 X *g* for 1 min. The supernatant was transferred to another tube and then stored at 2-8°C.

3.10 PCR-Based Genotyping of the Virulence Genes and Gel Electrophoresis

3.10.1 *vac*A and *cag*A Genotyping

About 3 *u*l of the supernatant containing the DNA template was added in 25 *u*l volumes containing 2.5 pmol of primers VAG-F and VAG-R, 25 pmol of primers VA1-F and VA1-R, 10 pmol of primers cag5c-F and cag3c-R (Table 1), 200 µM each dNTP is in 10 mM Tris-HCl, (pH 9.0 at room temperature), ~2.5 units of puReTaq DNA polymerase (GE Healthcare UK Limited), 50 mM KCl, and 1.5 mM of MgCl2 in a reaction standard PCR buffer (GE Healthcare UK Limited). Products were amplified under the following conditions: 3 min at 94°C for initial denaturation followed by 35 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C, with a final round of 10 min at 72°C, in a thermal cycler. All the PCR products were electrophoresed in 1.5% gel and visualized under UV light. The i region was genotyped using two independent PCRs with a universal forward primer (VacF1) and different i region type-specific reverse primers (C1R and C2R), as was described elsewhere (Rhead *et al.*, 2007). C1R and C2R primers anneal with the i1 and i2 *vacA* alleles, respectively.

Table 3.1: List of Primers used

Gene	Primer	Primer Sequence (5'-3')	Product size (bp)	Reference
***vac*As1/s2**	VAI-F	ATGGAAATACAACAAACACAC	s1, 259	Atherton *et al.*, 1995.
	VAI-R	CTGCTTGAATGCGCCAAAC	s2, 286	
***vac*Am1/m2**	VAG-F	CAATCTGTCCAATCAAGCGAG	m1, 567	
	VAG-R	GCGTCAAAATAATTCCAAGG	m2, 645	
vacA i1	C1R	TTAATTTAACGCTGTTTGAAG	426	Rhead *et al.*, 2007.
	VacF1	GTTGGGATTGGGGGAATGCCG		
vacA i2	C2R	GATCAACGCTCTGATTTGA	432	
	VacF1	GTTGGGATTGGGGGAATGCCG		
***cag*A**	cag5c-F	GTTGATAACGCTGTCGCTTC	350	Chattopadhyay *et al.,* 2004
	cag3c-R	5GGGTTGTATGATATTTTCCATAA		

3.10.2 Amplification of the 3-Variable Region of the *cag*A Gene and Determination of the EPIYA Pattern.

The following primers were used for amplification of the 3-variable region of the *cag*A gene; CAG1: 5-ACCCTAGTCGGTAATGGGTTA-3and CAG2: 5-GTAATTGTCTAGTTTCGC-3 as described elsewhere (Yamaoka *et al.*, 1998). The reaction mixture consisted of 0.5mMof each primer, 1X PCR buffer, 1.5mMMgCl2, sterile water, 0.2mM of each deoxynucleotide, 1.25 μL Taq DNA polymerase and 3 μL DNA in a final volume of 25 μL. The amplification conditions were initial denaturation at 95°C for 2min, followed by 35 cycles of denaturation at 95°C for 1min, annealing at 56°C, and extension at 72°C for 1 min, with a final extension at 72°C for 10min. PCR

was carried out in a thermo cycler Gene Amp PCR system 9700 (Applied Biosystems). Products of 500 to 850 bp were obtained depending on the type and number of repeats of the EPIYA-C motif in the *cag*A gene. The PCR products were separated by electrophoresis on 2% Agarose gel and stained with Ethidium bromide and visualized under a UV transilluminator. This procedure allowed for detection of multiple EPIYA motif patterns. For EPIYA-D alone; a reaction mixture containing 0.2mM concentrations of each deoxynucleoside triphosphate, a 0.4 nM concentration of the forward primer (cagA28F 5-TTCTCAAAGGAGCAATTGGC-3) , a 0.08 nM Concentration of the reverse primer (cagA-pD (R) 5-TTGATTTGCCTCATCAAAATC-3), 0.05 U of *Taq* DNA polymerase/ul, and 3ul of genomic DNA in buffer (10 mM Tris-HCl [pH 8.3], 1.5 mM magnesium chloride, 50 mM potassium chloride) was incubated at 95°C for 90s, followed by 35 cycles at 95°C for 30s, 57°C for 60s, and 72°C for 30s and a final extension at 72°C for 5 min. PCR products were separated on a 1.5% (wt/vol) agarose gel.

3.11 Data Management and Analysis

Data collection forms were used for recording of data in the hospital. Data analysis was done using SPSS version 17 (SPSS inc., Chicago, IL) program for windows. The association of the specific virulence-associated bacterial genotype and EPIYA motif with the clinical outcome of *H. pylori* infection was assessed using Chi-square (χ^2) or Fisher's exact tests to compare proportions for categorical variables. A value of $p < 0.05$ was considered statistically significant. Odds ratio was estimated and corresponding 95% confidence intervals were estimated while non-parametric correlations between the genotypes and severity of disease were assessed by use Kendall's tau_b test.

3.12 Ethical Considerations

1. Permission to carry out the study was obtained from Kenyatta National Hospital Scientific and Ethical Review committee. It was conducted according to the ethical guidelines of the declaration of Helsinki, 2000 (The International Response to Helsinki VI – The WMA's Declaration of Helsinki on Ethical Principles for Medical Research Involving Human Subjects, as adopted by the 52nd WMA General Assembly, Edinburgh, 2000.)
2. Consent. Patients were enrolled into the study only after giving informed consent (Appendix 1).
3. Confidentiality. All information obtained about the patients was handled with utmost confidentiality and only used for intended purposes.
4. Risks. Endoscopy is a safe procedure and when performed by a physician with specialized training in these procedures, the complications are extremely rare. They may include localized irritation, reaction to the medication or sedatives used and bleeding may occur at the site of a biopsy. There were no risks to patients in the study as we used the same biopsy used for diagnosis by the Doctor for our DNA analysis
5. Benefits. The patient got results for the tests undertaken.

CHAPTER FOUR

RESULTS

4.1 Demographic Characteristics

A total of 127 patients were enrolled into the study having met the predetermined criteria. These included 66 (52%) males and 61 (48%) females. The average age of the patients was 44.87 years. The representation per age group was 4 (3.15%) patients under 20yrs; 52 (40.94%) between 20 and 40 years; and 71 (55.91%) patients were above 40 years.

4.2 Endoscopic Findings

Gastritis was the single highest clinical findings at 63.8% of all the enrolled patients followed by Gastro-Oesophageal Reflux Disease (GERD) 13.4%, non-ulcer dyspepsia (NUD) 11.8%, peptic ulcer 9.4% and gastric cancer 7.9%. Gastric polyps, gastric nodules, gastrophathy and pre-pyloric mass were rare at 0.01% as shown in the Table 4.1.

Table 4.1: Frequencies of Various Endoscopic findings in the Enrolled Patients

Endoscopic Findings	Frequency (n)	% age
Gastritis	81	63.8
Gastro esophageal Reflux Disease	17	13.4
Normal	15	11.8
Gastric Cancer	10	7.9
Gastric Ulcer	7	5.5
Duodenal Ulcer	5	3.9
Gastric Outlet Obstruction	3	2.4
Duodenitis	2	1.6
Hiatus Hernia	2	1.6
Gastric polyps, Gastric Nodules, Gastrophathy, Pre-pyloric mass,	Each 1	Each 0.01

Key: *n = number of occurrences*

4.3 *Helicobacter pylori* Infection.

A total of 84 (66.14%) of 127 patients were positive by histology and 80 (62.99%) by molecular diagnosis (criteria**:** presence of at least three genotypes/alleles). Figure 4.1 shows a histopathology slide with *H. pylori* bacteria. For all associations with clinical outcome only those positive by molecular diagnosis were used.

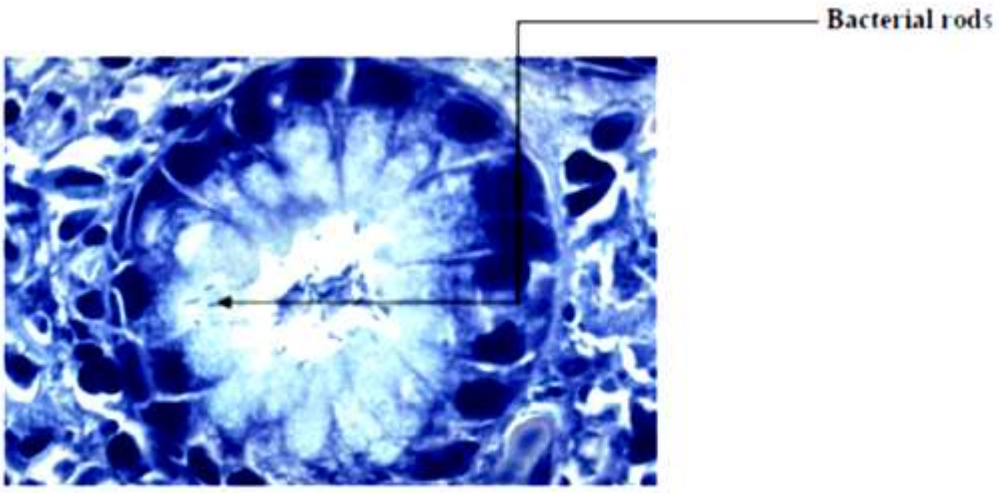

Figure 4.1: Photograph of the Histopathology Slide showing the Presence of H. pylori Bacteria Rods

4.3.1 Prevalence of *H. pylori* by age and sex in the Enrolled Patients.

Patients aged 40 and above years had highest *H. pylori* prevalence (62.5%); followed by those aged 20-39 years (64%); and lowest those under 20 years (50%) as shown in Table 4.2. Male patients had a lower prevalence at 59.01% against female patients at 67.21%. However, neither was there any significant association between *H. pylori* infection and age ($p < 0.083$) nor sex ($p < 0.34$).

Table 4.2: Prevalence of *H. pylori* by age and sex in the Enrolled Patients

Age in years /Sex (n)	Number positive	% age	P value
Under 20 Years (4)	2	50	0.849041
20-39 Years (48)	30	62.5	
40 and Above Years (75)	48	64	
Female (61)	41	67.21	0.343562.
Male (66)	39	59.09	

Key: *n = number of occurrences*

4.3.2 Prevalence of H. pylori in different Disease Conditions.

All the patients diagnosed with duodenal ulcer were positive for *H. pylori* infection while it was positive in 74.4% patients with gastric ulcer. Though the association was not significant ($p < 0.0870$), 69.1% of patients with gastritis were *H. pylori* positive implying a significant risk to the development of these pathologies. Significantly, ($p < 0.0015$) GERD was associated with *H. pylori* infection while 60% of patients diagnosed with gastric cancer were *H. pylori* positive as shown in Table 4.3.

Table 1.3: Prevalence of *H. pylori* in different Disease Conditions

Pathology (n)	Number Positive (n)	% age	P value
Gastritis (81)	56	69.1	0.0870
Duodenal Ulcer (5)	5	100	0.1567
Gastric Ulcer (7)	5	71.4	1.000
Peptic Ulcer (12)	10	83.3	0.2080
GERD (16)	4	25	0.0015
Gastric Cancer (10)	6	60	1.000

Key: *n = number of occurrences*

4.3.3 Distribution of Various Pathologies in the Gender among the *H. pylori* Positive Patients

There was no significant association between the distributions of various pathologies with gender. However, percentage distribution of peptic ulcer, gastric cancer and intestinal metaplasia was higher among the *H. pylori* positive males than females. Gastritis was highly distributed among the *H. pylori* positive females (75.61%) than males (65.54%) as shown in Table 4.4.

Table 4.4: Occurrence of Various Pathologies in the Gender among the *H. pylori* Positive Patients

Pathology	Gender (n)	Number positive	% age	P value
Peptic Ulcer	Female (41)	4	9.76	0.3431
	Male (39)	7	17.95	
Gastritis	Female (41)	31	75.61	0.2644
	Male (39)	24	61.54	
Gastric Cancer	Female (41	4)	9.76	0.5133
	Male (39)	6	15.38	
Intestinal Metaplasia	Female (41)	11	26.83	0.8870
	Male (39)	12	30.77	

Key: *n = number of occurrences*

4.3.4 Distribution of Pathologies in various age groups among the *H. pylori* Positive Patients

Table 4.5 shows that gastritis was significantly ($p < 0.00001$) associated with age, with 75.76% of patients aged below 40 years of age having a higher distribution of the pathology than those aged 40 years and above (63.83%). Though the association was not significant those aged 40 years and above had peptic ulcer ($p < 0.723$), intestinal metaplasia ($p < 0.080$) and gastric cancer ($p < 0.440$) distributed higher than those aged below 40 years of age.

Table 4.5: Distribution of Pathologies in various age groups among the *H. pylori* positive patients

	Less or = 40 Years (n = 33)		> 40 years (n = 47)		P value
	Frequency (n)	% age	Frequency (n)	% age	
Peptic Ulcer	4	12.12	7	14.89	0.723
Gastritis	25	75.76	30	63.83	0.001.
Gastric Cancer	3	09.09	7	14.89	0.440
Intestinal Metaplasia	6	18.18	17	36.17	0.080

Key: *n = number of occurrences*

4.3.5 Distribution of vacA and cagA Genotypes.

All the *H. pylori* positive samples had *vac*A gene as compared to 48.75% which had *cag*A gene. The most occurring alleles were *vac*A m2, i2 and s2 at 65%, 52% and 49%, respectively. The *vac*A m1, i1 and s1were lowly distributed in this study as shown in Table 4.6. The most occurring pairs of alleles were *vac*A i2/m2 (50%) and s2/m2 (45%). The *vac*A s1/m1 and s/1i1 were distributed at 30% and 28% among all the positive samples. The *vac*A s2/m1 combination was completely absent. Considering the multiple combinations, s2/m2/i2 was the most common multiple combination at 41.25% while s1/m1/i1 was distributed at 23.75% in all the *H. pylori* positive samples.

Table 4.6: Distribution of *vac*A Genotypes and *cag*A gene among the Positive Samples

Genotype	Number (n = 80)	% age	Genotype	Number (n = 80)	%	Genotype	Number (n= 80)	% age
*vac*As1	36	45	*vac*A s1/m1	22	28	*vac*A s1/m1/i1	19	23.75
*vac*As2	39	48.8	*vac*As1/i1	24	30	*vac*A s1/m/1i2	3	3.75
*vac*Am1	22	27.5	*vac*A s1/m2	15	19	*vac*A s1/m2/i1	6	7.5
*vac*Am2	52	65	*vac*A s1/i2	9	11	*vac*As1/m2/i2	7	8.75
*vac*Ai1	30	37.5	*vac*A s2/m1	0	0	*vac*A s2/m1/i1	0	0
*vac*Ai2	42	52.5	*vac*A s2/m2	36	45	*vac*As2/m1/i2	0	0
*cag*A	39	48.8	*vac*A i1/m2	9	11	*vac*As2/m2/i1	3	3.75
			*vac*A i2/m2	40	50	*vac*A s2/m2/i2	33	41.25

4.4 Associations of pathologies with various genotypes

A significant association (all at $p < 0.05$) between *vac*A m1, v i1, and s1 with peptic ulcer, intestinal metaplasia and gastric cancer was established. The *vac*A i2 was not significantly ($p < 0.43$) associated with gastric cancer. However, *vac*A s2 and m2 were significantly (all at $p < 0.05$) associated with gastritis as shown in Table 4.7. In this study, *cag*A gene had no significant association (all at $p > 0.05$) with any of the pathologies investigated despite being established in 54.4% and 60% of patients diagnosed with peptic ulcer and gastric cancer.

Table 4.7: Association between specific *H. pylori* Genotypes and Gastric Pathologies

Gastritis (55)					**Duodenal Ulcer (5)**				
Genotype n)	P value	OR	95% C.I OR			P value	OR	95% C.I OR	
*vac*A s1 (18)	0.002	0.189	0.067	0.535	*vac*A s1 (5)	0.011	All vacA s1 Positive		
*vac*A s2 (33)	0.004	4.750	1.638	13.777	*vac*A s2 (0)	-	All vac s2 Negative		
*vac*A m1 (22)	0.000	0.134	0.045	0.397	*vac*A m1 (4)	0.027	12.667	1.329	120.716
*vac*A m2(41)	0.010	3.727	1.377	10.090	*vac*A m2(1)	0.062	0.118	0.012	1.110
*vac*A i1(15)	0.006	0.250	0.092	0.677	*vac*A i1(5)	0.003			
*vac*A i2(33)	0.50	2.667	1.002	7.096	*vac*A i2(0)	-			
*cag*A(26)	0.695	0.828	0.321	2.132	*cag*A(3)	0.606	1.625	0.257	10.290
Gastric Ulcer (6)					**Pepticulcer (11)**				
	P value	OR	95% C.I OR			P value	OR	95% C.I OR	
*vac*A s1 (5)	0.084	6.935	0.771	62.351	*vac*A s1 (10)	0.009	16.538	2.000	136.756
*vac*A s2 (0)	0.013	0.473	0.372	0.602	*vac*A s2 (0)	-	-		
*vac*A m1 (2)	0.740	1.350	0.229	7.951	*vac*A m1 (6)	0.039	3.975	1.070	14.760
*vac*A m2(3)	0.430	0.510	0.096	2.714	*vac*A m2(4)	0.041	0.250	0.066	0.940
*vac*A i1(4)	0.146	3.692	0.633	21.530	*vac*A i1(9)	0.005	10.286	2.044	51.752
*vac*A i2(1)	0.103	0.161	0.018	1.446	*vac*A i2(1)	0.013	0.068	0.008	0.564
*cag*A a(3)	0.949	1.056	0.200	5.574	*cag*A(6)	0.679	1.309	0.365	4.696
Intestinal Metaplasia (23)					**Gastric Cancer (10)**				
	P Value	OR	95% C.I OR			P value	OR	95% C.I OR	
*vac*A s1 (17)	0.002	5.667	1.922	16.710	*vac*A s1 (9)	0.014	14.333	1.718	119.568
*vac*A s2 (6)	0.013	0.257	0.088	0.748	*vac*A s2 (0)	0.001	0.403	0.341	0.576
*vac*A m1 (11)	0.012	3.833	1.342	10.952	*vac*A m1 (6)	0.022	5.062	1.270	20.177
*vac*A m2(10)	0.012	0.275	0.100	0.757	*vac*A m2(3)	0.022	0.184	0.043	0.780
*vac*A i1(13)	0.029	3.059	1.124	8.322	*vac*A i1(7)	0.034	4.768	1.28	20.156
*vac*A i2(8)	0.047	0.361	0.132	0.989	*vac*A i2(2)	0.43	0.188	0.037	0.948
*cag*A(11)	0.916	0.949	0.360	2.502	*cag*A a6)	0.450	1.682	0.436	6.483

Key: *n = number of occurrences*

4.4.1 Association between specific pairs of *H. pylori* genotypes and Gastric Pathologies

This study further assessed the association between specific pairs of *H. pylori* genotypes and gastric pathologies. The *vac*A s2/m2, s2/i2 and m2/i2 were significantly ($p < 0.002$; $p < 0.009$; and $p < 0.024$, respectively) associated with gastritis at as shown in Table 4.8. All patients with duodenal ulcers were positive for *vac*A s1/m1 and s1/i1. However, due to fewer numbers of duodenal ulcers occurrence the odds ratio could not be established. When *cag*A was paired with *vac*A s1, m1 and i1, a strong association ($P<0.05$) was evidenced with peptic ulcer with an odds ratio of 11, 6.3 and 11.82, respectively. The *vac*A s1/m1 was highly associated with intestinal metaplasia ($P < 0.001$; OR, 6.33) and gastric cancer (P 0.004, OR, 16.13) almost a similar case with *vac*A s1/i1 ($P <0.05$). Both pairs were significantly ($P < 0.001$) associated with gastric cancer. The *cag*A gene was only significantly associated with gastric cancer when paired with *vac*As1 ($P <0.031$, OR, 11). The *vac*A s1/m2 did not significantly associate with any of the pathologies as shown in Table 4.8 (A-F).

Table 4.8, A: Association between specific pairs of H. pylori Genotypes and Gastric Pathologies

Gastritis (n = 55)				
Genotype pair (n)	**P value**	**OR**	**95% C.I OR**	
*vac*As1/m1 (8)	.001	.11	.03	.035
*vac*A s1/m2 (10)	.619	.33	.03	3.58
*vac*A s1/i1 (9)	.001	.14	.04	.43
*vac*A s1/i2 (6)	.285	.2	.02	2.39
*vac*A s1/*cag*A (8)	.019	.17	.04	.7
*vac*A s2/m1(0)	-	-	-	-
*vac*A s2/m2 (30)	.002	6.36	1.96	20.71
*vac*A s2/i1 (3)	.541	None Gastritis Negative		
*vac*A s2/i2 (27)	.009	4.5	1.46	13.84
*vac*A s2/*cag*A (15)	.087	4.09	.89	18.72
*vac*A m1/i1(7)	.001	.12	.04	.4
*vac*A m1/i2 (1)	.169	.13	.01	1.87
*vac*A m1/*cag*A (5)	.01	.14	.03	.61
*vac*A m2/i1(6)	1.0	.8	.09	6.85
*vac*A m2/i2(33)	.024	4.04	1.32	12.41
*vac*A m2/*cag*A(18)	.095	3.43	.87	13.52
*vac*A i1/*cag*A(9)	.068	.22	.05	.95
*vac*A i2/*cag*A(17)	.332	2.09	.55	7.91
*vac*A m2/i2(1)	.005	.2	.02	2
*vac*A m2/*cag*A(2)	.609	.52	.07	4.18
*vac*A i1/*cag*A(2)	.544	3.71	.31	44.66
*vac*A i2/*cag*A(1)	.334	.29	.03	2.99

Key: *n = number of occurrences*

Table 4.8, B: Association between specific pairs of H. pylori Genotypes and Gastric Pathologies

Duodenal Ulcer (n = 5)				
Genotype pair (n)	**P value**	**OR**	**95% C.I OR**	
*vac*A s1/m1 (4)	.011	None DU Negative		
*vac*A s1/m2 (1)	1.0	None DU Negative		
*vac*A s1/i1 (5)	.007	All s1/i1 Positive		
*vac*A s1/i2 (0)	1.0	None s1/i2 Positive		
*vac*A s1/*cag*A (3)	.077	None s1/cagA Positive		
*vac*A s2/m1(0)	-	-	-	-
VacA s2/m2 (0)	.024	All s2/m2 Negative		
*vac*A s2/i1 (0)	1.0	None s2/i1 Positive		
*vac*A s2/i2 (0)	.024	None s2/i2 Positive		
*vac*A s2/*cag*A (0)	.487	None s2/cagA Positive		
*vac*A m1/i1(4)	.005	All DU Positive		
*vac*A m1/i2 (0)	1.0	None m1/i2 Positive		
*vac*A m1/*cag*A (2)	.074	No DU Negative		
*vac*A m2/i1(1)	1.0	No DU Negative		
*vac*A m2/i2(0)	.021	None m2/i2 Positive		
*vac*A m2/*cag*A(1)	.550	.29	.02	3.52
*vac*A i1/*cag*A(3)	.045	No DU Negative		
*vac*A i2/*cag*A(0)	.233	None i2/cagA Positive		

Key: *n = number of occurrences*

Table 4.8, C: Association between specific pairs of H. pylori Genotypes and Gastric Pathologies

Gastric Ulcer (n = 6)				
Genotype pair (n)	**P value**	**OR**	**95% C.I OR**	
*vac*A s1/m1 (2)	.256	4.3	.32	50.25
*vac*A s1/m2 (3)	1.0	1.5	.13	17.67
*vac*A s1/i1 (4)	.069	7.4	.77	70.76
*vac*A s1/i2 (1)	1.0	1.25	.07	23.26
*vac*A s1/*cag*A (3)	.303	4.4	.42	46.43
*vac*A s2/m1(0)	-	-	-	-
*vac*A s2/m2 (0)	.064	All s2/m2 Negative		
*vac*A s2/i1 (0)	1.0	None s2/i1 Positive		
*vac*A s2/i2 (0)	.024	None s2/i2 Positive		
*vac*A s2/*cag*A (0)	.231	None s2/cagA Positive		
*vac*A m1/i1(2)	.573	2.65	.34	20.31
*vac*A m1/i2 (0)	1.0	None m1/i2 Positive		
*vac*A m1/*cag*A (1)	1.0	1.32	0.11	16.04
*vac*A m2/i1(2)	1.0	1.71	.12	23.94
*vac*A m2/i2(1)	.005	.2	.02	2
*vac*A m2/*cag*A(2)	.609	.52	.07	4.18
*vac*A i1/*cag*A(2)	.544	3.71	.31	44.66
*vac*A i2/*cag*A(1)	.334	.29	.03	2.99

Key: *n = number of occurrences*

Table 4.8, D: Association between specific pairs of H. pylori Genotypes and Gastric Pathologies

Peptic Ulcer (n = 11)				
Genotype pair (n)	**P value**	**OR**	**95% C.I OR**	
*vac*A s1/m1 (6)	.004	16.13	1.8	144.13
*vac*A s1/m2 (4)	.637	2.18	.2	24.21
*vac*A s1/i1 (9)	.000	22.2	2.58	190.84
*vac*A s1/i2 (1)	1.0	1.25	.07	23.26
*vac*A s1/*cag*A (6)	.031	11	1.18	102.38
*vac*A s2/m1(0)	-	-	-	-
*vac*A s2/m2 (0)	.001	All s2/m2 Negative		
*vac*A s2/i1 (0)	1.0	None s2/i1 Positive		
*vac*A s2/i2 (0)	.000	None s2/i2 Positive		
*vac*A s2/*cag*A (0)	.048	None s2/cagA Positive		
*vac*A m1/i1(6)	.006	10.38	1,87	57.71
*vac*A m1/i2 (0)	1.0	None m1/i2 Positive		
*vac*A m1/*cag*A (3)	.123	4.63	.7	33.61
*vac*A m2/i1(3)	.585	3	.24	37.67
*vac*A m2/i2(1)	.005	.07	.01	.61
*vac*A m2/*cag*A(3)	.225	.34	.06	1.82
*vac*A i1/*cag*A(5)	.021	11.82	1.23	113.23
*vac*A i2/*cag*A(1)	..095	0.15	.02	1.44

Key: *n = number of occurrences*

Table 4.8, E: Association between specific pairs of H. pylori Genotypes and Gastric Pathologies

Intestinal Metaplasia (n = 23)				
Genotype pair (n)	P value	OR	95% C.I OR	
*vac*A s1/m1 (11)	.001	6.33	1.91	21.03
*vac*A s1/m2 (5)	.616	3	.28	32.21
*vac*A s1/i1 (13)	.002	6.3	1.93	20.62
*vac*A s1/i2 (3)	.285	5	.42	59.66
*vac*A s1/*cag*A (9)	.043	4.75	1.15	19.65
*vac*A s2/m1(0)	-	-	-	-
*vac*A s2/m2 (5)	.008	1.52	.5	4.64
*vac*A s2/i1 (0)	.544	None s2/i1 Positive		
*vac*A s2/i2 (5)	.014	.23	.07	.75
*vac*A s2/*cag*A (2)	.067	0.19	.03	1.05
*vac*A m1/i1(10)	.014	4.69	1.47	14.92
*vac*A m1/i2 (1)	1.0	1.4	.1	19.01
*vac*A m1/*cag*A (6)	.137	3.43	.84	14.05
*vac*A m2/i1(3)	1.0	.67	.09	5.13
*vac*A m2/i2(7)	.024	.25	.08	.76
*vac*A m2/*cag*A(5)	.075	.25	.06	1.05
*vac*A i1/*cag*A(7)	.178	2.72	.71	10.41
*vac*A i2/*cag*A(4)	.185	.36	.09	1.46

Key: *n = number of occurrences*

Table 4.8, F: Association between specific pairs of H. pylori Genotypes and Gastric Pathologies

Gastric Cancer (n = 10)				
Genotype pair (n)	P value	OR	95% C.I OR	
*vac*A s1/m1 (6)	.004	16.13	1.8	144.58
*vac*A s1/m2 (3)	1.0	1.5	.13	17.67
*vac*A s1/i1 (7)	.004	15.24	1.74	133.78
*vac*A s1/i2 (6)	.566	2.86	.21	37.99
*vac*A s1/*cagA* (6)	.031	11	1.18	102.38
*vac*A s2/m1(0)	-	-	-	-
*vac*A s2/m2 (0)	.001	All s2/m2 Negative		
*vac*A s2/i1 (0)	1.0	None s2/i1 Positive		
*vac*A s2/i2 (0)	.002	None s2/i2 Positive		
*vac*A s2/*cag*A (0)	.107	None s2/*cag*A Positive		
*vac*A m1/i1(5)	.018	8.04	1.4	46.06
*vac*A m1/i2 (1)	.470	2.67	.18	39.65
*vac*A m1/*cagA* (6)	.137	3.43	.84	14.05
*vac*A m2/i1(2)	1.0	.71	.07	6.92
*vac*A m2/i2(1)	.013	.09	.01	.76
*vac*A m2/*cag*A(2)	.329	.32	.05	2.19
*vac*A i1/*cag*A(4)	.178	8.67	.87	86.06
*vac*A i2/*cag*A(2)	.412	.43	.07	2.61

Key: *n = number of occurrences*

4.4.2 Correlation between the *cag*A and the *vac*A genotypes with Severity of various Pathologies.

The severity of various pathological outcomes was associated with various genotypes as shown in Table 4.9. The degree of inflammatory activity according to the density of neutrophils in gastric mucosa was not significantly correlated with any genotype while intensity of the superficial epithelial damage significantly correlated with *vac*A s1 (p= 0.002), m1 (p = 0.023), and i1 (p = 0.008). The *vac*A s1 was the only genotype that significantly correlated with degree of chronic inflammatory infiltrate in the gastric mucosa (p = 0.016) and severity of gastritis (p = 0.019). The intensity of intestinal metaplasia was graded according to the amount of glandular tissue replaced by intestinal– type epithelium and in this case *vac*A s1,m1 and i1 were significantly associated with its severity at, p = 0.001, p = 0.010 and p = 0.023, respectively. The *cag*A gene was not significantly (all at p > 0.05) correlated with severity of any of these disease conditions.

Table 4.9: Correlation between the cagA and the vacA Genotypes with Severity of various Pathologies

Severity of Gastritis						**Degree of Inflammatory activity**					
Genotype (n)	Severity				*p* value	Genotype (n)	Severity				*p* value
	0	***1***	***2***	***3***			***0***	***1***	***2***	***3***	
*vac*A s1 (36)	3	12	21	0	.019*	*vac*A s1 (36)	8	13	15	0	.064
*vac*A s2(39)	3	26	9	1	.032^{-}	*vac*A s2(39)	9	24	6	0	.146^{-}
*vac*A m1(22)	2	7	13	0	.106	*vac*A m1(22)	5	6	11	0	.059
*vac*A m2(52)	5	28	9	1	.297^{-}	*vac*A m2(52)	13	29	10	0	.095^{-}
*vac*A i1(30)	1	12	16	0	.073	*vac*A i1(30)	4	14	11	0	.053
*vac*A i2(42)	4	24	13	1	.201^{-}	*vac*A i2(42)	12	21	9	0	.146
*cag*A (39)	6	15	18	0	.700^{-}	*cag*A (39)	9	18	12	0	.858
Intensity of Epithelial Damage						**Degree of Mucosa Infiltration**					
Genotype (n)	Severity				*p* value	Genotype (n)	Severity				*p* value
	0	***1***	***2***	***3***			***0***	***1***	***2***	***3***	
*vac*A s1 (36)	10	11	15	0	.023*	*vac*A s1 (36)	7	14	14	1	.016*
*vac*A s2(39)	13	23	3	0	.035^{-}	*vac*A s2(39)	13	20	5	1	.036^{-}
*vac*A m1(22)	5	7	10	0	.026*	*vac*A m1(22)	4	9	9	0	.108
*vac*A m2(52)	18	26	8	0	.052^{-}	*vac*A m2(52)	16	24	11	1	.194^{-}
*vac*A i1(30)	4	15	10	0	.008*	*vac*A i1(30)	4	16	9	0	.138
*vac*A i2(42)	16	19	7	0	.070^{-}	*vac*A i2(42)	15	18	8	1	.068^{-}
*cag*A (39)	11	18	10	0	.560	*cag*A (39)	10	19	10	0	.917^{-}
Level of Intestinal Metaplasia						**Level of Atrophy**					
Genotype (n)	Severity				*p* value	Genotype (n)	Severity				*p* value
	0	***1***	***2***	***3***			***0***	***1***	***2***	***3***	
*vac*A s1 (36)	19	11	6	0	.001*	*vac*A s1 (36)	19	12	5	0	.001*
*vac*A s2(39)	33	6	0	0	.006^{-}	*vac*A s2(39)	33	6	0	0	.004^{-}
*vac*A m1(22)	11	8	3	0	.010*	*vac*A m1(22)	11	7	4	0	.011*
*vac*A m2(52)	42	8	2	0	.009^{-}	*vac*A m2(52)	42	9	1	0	.013^{-}
*vac*A i1(30)	16	9	4	0	.023*	*vac*A i1(30)	16	9	4	0	.013*
*vac*A i2(42)	34	7	1	0	.033^{-}	*vac*A i2(42)	34	7	1	0	.039^{-}
*cag*A (39)	28	8	3	0	.932^{-}	*cag*A (39)	28	7	4	0	.928^{-}

Key: *n = number of occurrences; * Significant positive correlation; ¯ Negative correlation Severity: 0 = none; 1 = mild; 2 = moderate; 3 = severe*

4.5 Distribution of EPIYA Motifs Pattern

PCR amplified products from all the *cag*A positive strains showed varied patterns in the variable region of the CagA protein as shown in a representative UV film in Figure 4.2. The most occurring EPIYA pattern was the ABC (56.41%) followed by ABCC at 43% and AB at 28.21%. The ABCCC pattern was rare (5.13%) as shown in the Table 4.10. There was no EPIYA ABD pattern.

Table 4.10: Distribution of EPIYA Motifs Patterns

Pattern	Number (n)	% age in *cag*A positive patients (39)	% age in all the *H. pylori* positive patients (80)	Strain type
ABD	0	0	0	East Asian
AB	11	28.21	13.75	-
ABC	22	56.41	27.5	Western
ABCC	17	43.59	21.25	Western
ABCCC	2	5.13	2.5	Western

Key: *n = number of occurrences*

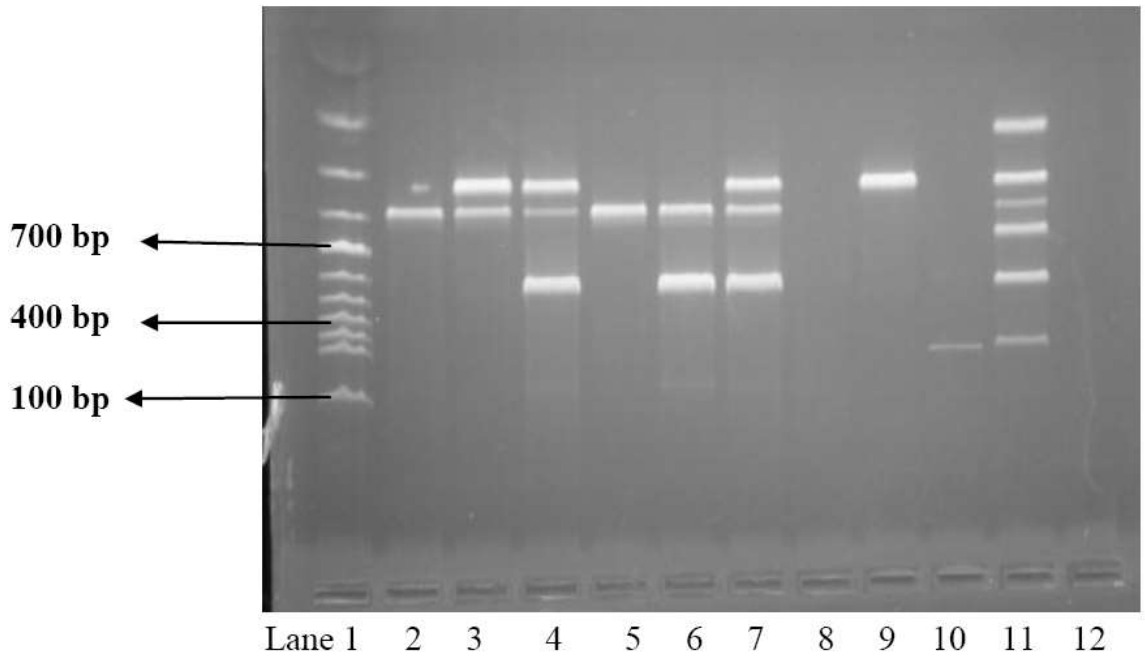

Figure 4.2: AUV Film showing the various EPIYA Motif Patterns in a Sample of Patients

Key: *Lane 1, 100bp standard; Lane 2&5, ABCC; Lane 3, ABCC &ABCCC; Lane 4&7, AB, ABCC & ABCCC; Lane 6, AB &ABCC, Lane 8, no EPIYA pattern; Lane 9, ABCC; Lane 11, All patterns available; Lane 12, negative control*

4.5.1 Association between the EPIYA Motif Patterns and Gastric Pathologies

Increase in the number of EPIYA C repeats was not significantly (p=0.768) associated with gastric ulcer and duodenal ulcer. EPIYA pattern with more than one C was significantly associated with Intestinal Metaplasia (p=0.045, OR 3.021, 95% CI 1.026 - 8.900), and gastric cancer (p = 0.008, OR=6.577, 95% CI. 1.620 - 26.704) as shown in Table 4.11.

Table 4.11: Association between the EPIYA Motif Patterns and Gastric Pathologies

Gastritis (n=55)					**Duodenal Ulcer (n=5)**				
EPIYA Motif pattern (n)	P value	OR	95% C.I OR		**EPIYA Motif pattern (n)**	P value	OR	95% C.I OR	
EPIYA AB (9)	0.324	2.250	0.449	11.2777	EPIYA AB (1)	0.678	1.625	0.165	16.050
ABC (13)	0.287	0.550	0.197	1.536	ABC(2)	0.612	1.833	0.285	11.778
EPIYA Less than 2 C or none (22)	0.736	0.848	0.326	2.208	EPIYA Less than 2 C or none(3)	0.390	2.250	0.355	14.279
EPIYA more than 1C (14)	0.596	1.366	0.431	4.3208	EPIYA more than 1C(1)	0.839	0.7992	0.083	7.545
Gastric Ulcer (n=6)					**Peptic Ulcer (n=11)**				
EPIYA Motif pattern (n)	P value	OR	95% C.I OR		**EPIYA Motif pattern (n)**	P value	OR	95% C.I OR	
EPIYA AB(0)	0.309	0.851	0.774	0.936	EPIYA AB (1)	0.632	0.590	0.068	5.127
ABC(2)	0.665	1.350	1.229	7.957	ABC (4)	0.483	1.619	0.424	6.188
EPIYA Less than 2 C or none(2)	0.682	0.694	0.119	4.027	EPIYA Less than 2 C or none (5)	0.761	1.220	0.339	4.391
EPIYA more than 1C .(2)	0.570	1.676	0.282	9.958	EPIYA more than 1C(3)	0.768	1.242	0.294	5.242
Intestinal Metaplasia (n=23)					**Gastric Cancer (n=10)**				
EPIYA Motif pattern (n)	P value	OR	95% C.I OR		**EPIYA Motif pattern (n)**	P value	OR	95% C.I OR	
EPIYA AB (1)	0.153	0.214	0.026	1.1775	EPIYA AB (0)	-			
ABC (10)	0.042	2.885	1.018	8.175	ABC (2)	0.719	0.625	0.122	3.203
EPIYA Less than 2 C or none (11)	0.449	1.458	0.549	3.872	EPIYA Less than 2 C or none (2)	0.162	0.315	0.062	1.589
EPIYA more than 1C (9)	0.045	3.021	1.026	8.900	EPIYA more than 1C (6)	0.008	6.577	1.620	26.704

***Key**: n = number of occurrences*

4.5.2 Correlation between the EPIYA Motif Variants with Severity of various Pathologies

An evaluation was further done to establish whether each of the patterns correlated with the degree of various pathologies. EPIYA ABC significantly (P =0.039) correlated with increased degree of intestinal metaplasia. There was also significant correlation between the EPIYA patterns with more than one C repeat segment with increased degree of intestinal metaplasia (p=0.021). Because of the fewer EPIYA ABCCC (2) positive-samples no correlation was made with various pathologies. The degree of chronic inflammatory infiltrate in the gastric mucosa significantly correlated with EPIYA patterns with more than one C repeat segment (p=0.021) as opposed to the EPIYA patterns with one or no C repeat segment (p=0.095) as shown in Table 4.12. Surprisingly, neither of the EPIYA pattern significantly correlated with degree of inflammatory activity nor the degree of gastritis in this study.

Table 4.12: Correlation between the EPIYA Motif Variants with Severity of various Pathologies

Severity of Gastritis						**Degree of Inflammatory activity**					
Genotype (n)e	Severity				*p* value	Genotype (n)	Severity				*p* value
	0	***1***	***2***	***3***			***0***	***1***	***2***	***3***	
AB (11)	1	3	7	0	.442	AB (11)	3	5	3	0	.258-
ABC (22)	3	9	9	1	.811	ABC (22)	4	12	6	0	.680 -
ONE C OR NONE (33)	4	12	16	1	.451	ONE C OR NONE (33)	7	17	9	0	.677
MORE THAN ONE C(19)	0	10	8	1	.379	MORE THAN ONE C (19)	5	9	6	0	.902
ABCC (17)	0	8	8	1	.379	ABCC (17)	4	7	6	0	.902
ABCCC (2)	0	2	0	0	-	ABCCC (2)	0	2	0	0	-
Intensity of Epithelial Damage						**Degree of Mucosa Infiltration**					
Genotype (n)	Severity				*p* value	Genotype (n)	Severity				*p* value
	0	***1***	***2***	***3***			***0***	***1***	***2***	***3***	
AB (11)	3	7	1	0	.341 -	AB (11)	1	6	5	0	.089
ABC (22)	5	11	6	0	.357	ABC (22)	4	13	3	2	.597
ONE C OR NONE (33)	9	18	7	0	.866	ONE C OR NONE (33)	5	19	8	2	.095
MORE THAN ONE C (19)	8	6	5	0	.922	MORE THAN ONE C (19)	4	7	6	2	.021*
ABCC (17)	7	5	5	0	.922	ABCC (17)	3	6	6	2	.021*
ABCCC (2)	1	1	0	0	-	ABCCC (2)	1	1	0	0	-
Level of Intestinal Metaplasia						**Level of Atrophy**					
Genotype (n)	Severity				*p* value	Genotype (n)	Severity				*p* value
	0	***1***	***2***	***3***			***0***	***1***	***2***	***3***	
AB (11)	10	1	0	0	.115 -	AB	10	1	0	0	.015-
ABC (22)	12	7	3	0	.039	ABC	12	8	2	0	.060
ONE C OR NONE (33)	22	8	3	3	.445	ONE C OR NONE (33)	22	9	2	0	.047
MORE THAN ONE C (19)	12	4	3		.022*	MORE THAN ONE C (19)	12	7	2	0	.060
ABCC (17)	10	4	3	0	.022*	ABCC (17)	9	6	2	0	.060
ABCCC (2)	2	0	0	0	-	ABCCC (2)	2	0	0	0	-

Key: *n = number of occurrences; *Positive correlation; ¯ Negative correlation; Severity: 0 = none; 1 = mild; 2 = moderate; 3 = severe*

CHAPTER FIVE

DISCUSSION

5.1 Discussion

Gastritis, like in many other studies in Kenya was a more frequent gastro duodenal disease (63.8%) while gastric cancer remained rare, at 7.9% (Lwai-lume *et al.*, 2005; Kimang'a, 2013), despite the high prevalence of *H. pylori* infection. The prevalence of *H. pylori* was found to be 62.99% among the dyspeptic patients slightly lower than the earlier finding in Kenya (Kimanga *et al.*, 2010), which documented 67.5% in all age groups in dyspeptic patients. Other studies (Smith *et al.*, 2002; Sanz-Pelaez *et al.*, 2008) in other African countries found *H. pylori* prevalence levels of >90%. Larger proportions of those aged 40 years and above had more violent pathologies such as peptic ulcer disease, intestinal metaplasia, and gastric cancer than those aged less than 40 years. Similarly, Ogutu, *et al.*, (1998) reported the presence of gastric cancer in patients aged 50 years and above accounting for 17.4% of dyspeptic symptoms in this age group. All cases of duodenal ulcers were reported in patients aged below 40 years, similar to an earlier study (Ogutu, *et al.*, 1998).There was no association of *H. pylori* infection with age or sex in contrast to what was observed elsewhere (Zhu *et al.*, 2014). Therefore, this suggests other underlying host factors contribute to disease risk.

On analysis of *vac*A gene polymorphisms, the most occurring alleles were m2, i2 and s2 at 65%, 52% and 49%, respectively. These polymorphisms have been found to be less virulent (Meining *et al.*, 1997; Higashi *et al.*, 2002; Rhead *et al.*, 2007; Argent *et al.*, 2008). This study established that the more virulent *vac*A isoforms, m1, i1 and s1 (Meining *et al.*, 1997; Higashi *et al.*, 2002; Rhead *et al.*, 2007; Argent *et al.*, 2008;) to be lowly distributed in the Kenyan *H. pylori* strains, possibly giving an explanation of low occurrence of serious pathologies in Kenya. The distribution of the *vac*A pairs of alleles was also assessed. The most occurring pairs of alleles were i2/m2 (50%) and s2/m2 (45%). The s1/m1 and s1/i1 which are highly toxigenic (Atherton *et al.*, 1997; Rhead *et*

al., 2007; Argent *et al.*, 2008) were distributed at 30% and 28% among all the positive samples. This explains the lower incidence of dangerous pathologies in Kenya. The *vac*A s2/m1 combination was completely absent as was documented elsewhere in Africa (Letley *et al.*, 1999)

Significant association between *vac*A m1, i1, and s1 with peptic ulcer, intestinal metaplasia, atrophy and gastric cancer (all at $P < 0.05$) was established as documented elsewhere (Basso *et al.*, 2008).The less virulent polymorphisms, m2 and i2 (Meining *et al.*, 1997; Higashi *et al.*, 2002; Rhead *et al.*, 2007; Argent *et al.*, 2008;) were significantly (all at $P < 0.05$) associated with gastritis.

In this study, *cag*A gene was not significantly ($p < 0.917$) correlated with the severity of mucosa infiltration which is in concordance with an earlier study (Basso *et al.*, 2008), which associated *cag*A gene with more severe antral and corpus activity but not with the degree of inflammatory activity. The *vac*A s1, m1, and i1 were significantly (all at $P < 0.05$) correlated with the severity of intestinal metaplasia and intensity of epithelial damage. Possibly this is the first study in Kenya to correlate the various genotypes with the severity of gastro duodenal diseases. In this study, it was only the *vac*A s1 genotype that was significantly ($p < 0.016$) positively correlated with the intensity of mucosa infiltration. This was in agreement with another study (Basso *et al.*, 2008), which also in addition to *vac*A s1, the *vac*A m1 and i1 significantly correlated with intensity of mucosa infiltration.

Further, when *cag*A was paired with *vac*A s1 and i1 strong association ($P < 0.031$ and $P < 0.021$) was evidenced with peptic ulcer. The *vac*A s1/m1 was highly associated ($P < 0.001$) with intestinal metaplasia almost a similar case with *vac*A s1/i1 ($P < 0.002$). Both pairs were significantly ($P < 0.004$) associated with gastric cancer. Studies have shown a strong correlation between toxin activity and the pathogenecity of *H. pylori*, with the s1/m1 type of *vac*A being the most virulent in Western populations (Artherton *et al.*, 1995; Artherton *et al.*, 1997; Gunn *et al.*, 1998; van Doorn *et al.*, 1998; Wang *et al.*,

1998; Keates *et al*., 1999). In contrast, such an association was not observed when Asian subjects were studied (Yamaoka *et al*., 1997). The *cag*A gene was only significantly ($P < 0.031$) associated with gastric cancer when paired with *vac*A s1.

Many studies have shown infection with *cag*A –positive *H. pylori* strain to be closely associated with gastric cancer and peptic ulcers (Peek & Blaser, 2002; *Palli et al*., 2007). Nonetheless, the majority of the *H. pylori* infected patients do not develop serious diseases (Bosques-Padilla *et al*., 2003; Torres *et al*., 2005; Carmolinga-Ponce *et al*., 2008). The differences in the prevalence of *H. pylori* can be explained by differences in the diagnostic method used, age of patients, geographic area and the environmental health conditions in which people live or that the rate of infection is decreasing (Torres *et al*., 2005). In this study the prevalence of *cag*A gene was 48.75% lower than other reports in African countries (Kimanga*et al*., 2010; Breurec *et al*., 2012), probably giving a reason as to why there are rare virulent pathologies in Kenya. Further, *cag*A gene had no significant association (all at $P > 0.05$) with any of the pathologies despite being established in 54.4% and 60% of patients diagnosed with peptic ulcer and gastric cancer. This was in concordance with other studies done in western countries (Atherton *et al*., 1995; Blaser *et al*., 2004; Palli *et al*., 2007; Basso *et al*., 2008;) but in agreement with another study (Atherton *et al*., 1995). Studies have shown that *cag*A is polymorphic (Higashi *et al*., 2002) and the distribution of EPIYA motif patterns and combination differs geographically. Possibly, Kenya's CagA protein EPIYA motif patterns induce less pathological changes.

In this study, no EPIYA ABD pattern was observed indicating the absence East Asian *H. pylori* strain in Kenya as was found elsewhere in Africa (Breurec *et al*., 2012). The most occurring EPIYA pattern was the ABC (56.41%) followed by ABCC at 43% and AB at 28.21%. The predominance of ABC pattern mirrors a study (Chomvarin *et al*., 2012) among the dyspeptic patients in Northern Thailand. The presumed violent ABCCC pattern was rare (5.13%) as observed elsewhere (Breurec *et al*., 2012). This suggests that the Kenyan *H. pylori* are of Western type.

None of the gastric pathologies including peptic ulcer (P < 0.679) and gastric cancer (P < 0.45) was associated with the presence of *cag*A gene alone. This differs from many other studies that associated *cag*A gene with peptic ulcer and gastric cancer (Nomura *et al.*, 2002; Blaser *et al.*, 1995; Salih *et al.*, 2010). The lack of association correlates with another recent study in Kenya (Kimang'a *et al.*, 2013) that found no significant association of *cag*A gene with gastric pathologies. However, 60% and 54.5% of patients with gastric cancer and peptic ulcer, respectively had *cag*A-positive *H. pylori* strain indicating possible risk. Other pathologies had their distributions below a 50% mark.

In this study none of the EPIYA patterns was significantly associated with gastritis, duodenal ulcer or gastric ulcer. The EPIYA ABC was significantly associated with intestinal metaplasia (OR=2.885, 95% CI 1.018 -8.175, p=0.042) and not gastric cancer. Patients with more than one C repeat had an odds ratio (OR, 3.021) 2-fold of having intestinal metaplasia than those with one or no C segment (OR, 1.458). Analysis of gastric histopathology showed a significant correlation between the EPIYA motif pattern with more than one C repeat segment with increased degree of Intestinal metaplasia (P <0.021) as had been reported elsewhere (Basso *et al.*, 2008). Gastric cancer was significantly associated with samples with more than one C repeat (OR=6.577 95% CI 1.620-26.704, p=0.008). It has been documented that the number of EPIYA-C motifs influence the degree of virulence and oncogenic potential of cagA–positive *H. pylori* (Basso *et al.*, 2008). Studies have shown that an increase in the number of phosphorylation sites in the C-terminus of CagA protein is associated with the carcinogenic potential of *H. pylori* (Reyes-Leon *et al.*, 2007; Sicinschi *et a*l., 2010; Acosta *et al.*, 2010) Therefore, it is more likely that those patients with chronic gastritis infected with a *H. pylori* strain with *cag*A gene that encodes two or more EPIYA-C motifs (25.45%) are at higher risk of developing intestinal metaplasia and gastric cancer. Thus, determining the EPIYA motif pattern in CagA protein, rather than detecting *cag*A alone, would be a better marker for assessing the risk of serious gastric pathology (Argent *et al.*, 2004).

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

This study has shown that the dyspeptic patients are not inclined to any gender. However, most of these patients were over 40 years old. Further, there seems to be varied gastro-duodenal diseases in the patients presented but gastritis appeared in about two-thirds of the population. The prevalence of *H. pylori* infection could be decreasing if the findings of this study were compared to earlier studies. Most of the patients presenting with duodenal ulcers could be *H. pylori* positive as seen in this study. Gastro–esophageal reflux disease was a frequent gastro duodenal disease and was associated with *H. pylori* infection. Gastric cancer was a rare disease in this study population. In comparison, gastritis was highly distributed in females while gastric cancer and peptic ulcer was highly distributed in males.

This study has provided information on the genotypes of the Kenyan *H. pylori* strains. Despite the high prevalence of *H. pylori* infection, the less toxigenic *vac*A isoforms s2, m2 and i2 are highly distributed in Kenya. Further, it has shown that *cag*A-positive *H. pylori* infection is averagely prevalent in dyspeptic patients in Kenya and all the CagA protein EPIYA motif patterns were of Western type.

This study has further confirmed the potential of *vac*A s1, m1 and i1 to induce gastric cancer and peptic ulcer. Still, the *vac*A s1/m1 and s1/i1 are more virulent *vac*A subtypes and *cag*A was significantly associated with gastric cancer when paired with *vac*A s1. Infection by *H. pylori cag*A strains with multiple EPIYA C repeats were found to be associated with gastric cancer but not peptic ulcer; but the low prevalence of these strains might contribute to the low incidence of gastric cancer in this study population.

Thus, determining the EPIYA motifs in CagA protein, rather than detecting *cag*A gene alone, would be a better marker for assessing the risk of serious gastric pathology. Further, it's not the density of colonization that confers the severity of the disease but the specific genotypes. The possibility of the presence of unidentified strain or certain host or environmental factors that trigger *H. pylori*-induced cancer and peptic ulceration cannot be ruled out.

6.2 Recommendations

1. Determination of the EPIYA motifs in cagA protein should be used to assess the risk of serious gastric pathology rather than detecting *cag*A gene alone.
2. More studies should be carried out to help increase our understanding of bacterium-host interactions in colonization and disease and offer insight into the epidemiology of the disease hence increased disease surveillance and control benefit.
3. There is need to establish the environmental and behavioural factors that lead to varied distribution of various gastro duodenal diseases in different gender.
4. Detection of the EPIYA motif pattern of *H. pylori* even in patients with gastritis alone should be done since its more likely that those patients with gastritis infected with a *H. pylori* strain with *cag*A gene that encodes two or more EPIYA-C motifs are at higher risk of developing intestinal metaplasia and gastric cancer.
5. More research is required to affirm whether the presence of *H. pylori cag*A EPIYA motif patterns have a significant geographic variability, closely follow patterns of historical human migrations and what host factors determine the establishment of each strain.

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APPENDICES

Appendix 1: Consent form for persons aged 18 years and above and children under the age of 18 years who are able to understand the purpose of the study

Introduction

You are asked to participate in a medical research study on the clinical relevance of the various genotypes of *Helicobacter pylori* in patients with dyspepsia. *Helicobacter pylori* is a spiral, gram-negative micro-aerophilic bacterium that chronically infects the gastric mucosa of more than half of all people worldwide, and is a major cause of gastritis and peptic ulcer (PU) disease, and a risk factor for gastric adenocarcinoma and mucosa-associated lymphoid tissue lymphoma. The differences in disease outcome may be the result of host factors, environmental factors and differences in the prevalence or expression of bacterial virulence factors. The purpose of this consent form is to give you information that might help you to decide whether to participate in the study or not. You are allowed to ask questions related to the study and implications on your part

Purpose of the study

The aim of this study is to evaluate the clinical relevance of the various genotypes of *Helicobacter pylori* in patients with dyspepsia. The findings of this project will provide information on the genotypes of the Kenyan *H. pylori* strains and help us gain new insights into the population genetic structure of this important human pathogen and to learn if genotypes and gene polymorphisms implicated in the disease in the West as well as Eastern Asia are similarly disease associated in Kenya. Studies of *H. pylori* genotypes and gene polymorphisms from different well-separated human populations will help increase our understanding of bacterium-host interactions in colonization and disease. These findings will further help in coming up with research based evidence on the type of immunological tests to be employed in *H. pylori* infected patients in Kenya. This is

also as a partial fulfillment for award of a PhD degree in Medical Microbiology at the Institute of Tropical Medicine and Infectious Diseases, KEMRI/JKUAT.

Procedures to be followed

If you have been referred for endoscopy, due to disease conditions referred to us dyspepsia for assessment of organic disease, I shall ask you to allow me to use the results of your biopsy related testing methods and further use the same biopsies for genetic analysis.

Risks

Endoscopy is a safe procedure and when performed by a physician with specialized training in these procedures, the complications are extremely rare. They may include localized irritation, reaction to the medication or sedatives used and bleeding may occur at the site of a biopsy. There will be no risks to patients in this study as we will use the same biopsy used for diagnosis by the Doctor for our DNA analysis

Benefits

The patient will get results for all the tests undertaken except endoscopy and know the strain type of *H. pylori*

Assurance of confidentiality

Your name and other records about you will remain confidential and only used for intended purposes and will not appear when we present this study or publish its results. You will receive a copy of the consent form.

Right to refuse or withdraw

It is important that you understand the following general principles that will apply to all participants in the study:

1. Participation is entirely voluntary.

2. You may withdraw from this study at any time without penalty or loss of benefits.

Please feel free to ask any questions that you may have. Do you agree to participate?

Declaration

I acknowledge that this consent form has been fully explained to me in a language that I understand and agree to participate in the study. I understand that participation in this study is purely voluntary, and I hereby allow you to use my biopsies and endoscopy results for the purpose explained to me. I further understand that I will have made no crime to decline to the participation at any time without suffering any consequences such as denial of medication.

In case of need for further information about this study, please contact the investigator:

Lawrence Guantai, Tel: 0724 501 453, ITROMID/JKUAT/DTE, P.O. Box 60209-00200, **Nairobi** or the Secretary, Kenyatta National Hospital/University of Nairobi-Ethical Review Committee

P.O. Box 20723-00202 **Nairobi**

Participant's name: _____

Participant's signature or thumb print: _____ Date: _____

Study No.: _____

Name of witness: _____

Signature of witness: _____ Date: _____

Investigator's signature: _____ Date: _____

Appendix 2: Consent form for persons aged below 18 years and are unable to understand the purpose of the study

Introduction

You child is asked to participate in a medical research study on the clinical relevance of the various genotypes of *Helicobacter pylori* in patients with dyspepsia. *Helicobacter pylori* is a spiral, gram-negative micro aerophilic-bacterium that chronically infects the gastric mucosa of more than half of all people worldwide, and is a major cause of gastritis and peptic ulcer (PU) disease, and a risk factor for gastric adenocarcinoma and mucosa-associated lymphoid tissue lymphoma. The differences in disease outcome may be the result of host factors, environmental factors and differences in the prevalence or expression of bacterial virulence factors. The purpose of this consent form is to give you information that might help you to decide whether to participate in the study or not. You are allowed to ask questions related to the study and implications on your part

Purpose of the study

The aim of this study is to evaluate the clinical relevance of the various genotypes of *Helicobacter pylori* in patients with dyspepsia. The findings of this project will provide information on the genotypes of the Kenyan *H. pylori* strains and help us gain new insights into the population genetic structure of this important human pathogen and to learn if genotypes and gene polymorphisms implicated in the disease in the West as well as Eastern Asia are similarly disease associated in Kenya. Studies of *H. pylori* genotypes and gene polymorphisms from different well-separated human populations will help increase our understanding of bacterium-host interactions in colonization and disease. These findings will further help in coming up with research based evidence on the type of immunological tests to be employed in *H. pylori* infected patients in Kenya. This is also as a partial fulfillment for award of a PhD degree in Medical Microbiology at the Institute of Tropical Medicine and Infectious Diseases, KEMRI/JKUAT.

Procedures to be followed

If he/she has been referred for endoscopy, due to disease conditions referred to us dyspepsia for assessment of organic disease, I shall ask you to allow me to use the results of your biopsy related testing methods and further use the same biopsies for genetic analysis.

Risks

Endoscopy is a safe procedure and when performed by a physician with specialized training in these procedures, the complications are extremely rare. They may include localized irritation, reaction to the medication or sedatives used and bleeding may occur at the site of a biopsy. There will be no risks to patients in this study as we will use the same biopsy used for diagnosis by the Doctor for our DNA analysis

Benefits

The patient will get results for all the tests undertaken except endoscopy and know the strain type of *H. pylori*

Assurance of confidentiality

His/her name and other records about him/her will remain confidential and only used for intended purposes and will not appear when we present this study or publish its results. You will receive a copy of the consent form.

Right to refuse or withdraw

It is important that you understand the following general principles that will apply to all participants in the study:

1. Participation is entirely voluntary.

2. You may decide to withdraw your child from this study at any time without penalty or loss of benefits.

Please feel free to ask any questions that you may have. Do you agree for his/her participation?

Declaration

I acknowledge that this consent form has been fully explained to me in a language that I understand and agree for the participation of my child in this study. I understand that participation in this study is purely voluntary, and I hereby allow you to use his/her biopsies and endoscopy results for the purpose explained to me. I further understand that I will have made no crime to decline for his/her participation at any time without suffering any consequences such as denial of medication.

In case of need for further information about this study, please contact the investigator

Lawrence Guantai, Tel: 0724 501 453, ITROMID/JKUAT/DTE, P.O. Box 60209-00200, **Nairobi** or the Secretary, Kenyatta National Hospital/University of Nairobi-Ethical Review Committee, P.O. Box 20723-00202 **Nairobi**

Child's name: _____

Parent/guardian's Name_____

Parent's/guardian's signature or thumb print: _____ Date: _____

Study No.: _____

Name of witness: _____

Signature of witness: _____ Date: _____

Investigator's signature: _____ Date: _____

Appendix 3: Demographic Information.

Date……

Study subject No……. Name……

Name of your County.....

Criteria for enrolment:

- Read/understood/signed consent
- Past or present history of dyspepsia and have been referred for endoscopy
- Have not been on any antibiotics, PP1 or H_2 blockers within 4 weeks prior to presentation

ALL MUST BE MET TO PROCEED

Demographic Information.

Age in years……...

☐ 10-19 years

☐ 20 - 29 years

☐ 30 – 39 years

☐ 40 – 49 years

☐ 50 – 59 years

☐ ≥60 years

SEX: ☐ MALE ☐ FEMALE

Appendix 4: Dyspepsia Screening Form.

Mark (* the) condition presented as appropriate

- Bloating
- Abdominal pain
- Tenderness on palpation
- Discomfort or fullness
- Intestinal gas (flatulence)
- Heartburn
- Nausea
- Gastro esophageal reflux
- Liver disease,
- Difficulty in swallowing
- Loss of appetite,
- Parasitic infections,
- Gastrointestinal bleeding
- Persistent vomiting
- Peptic ulcer
- Gastric cancer
- Gallstones
- Oesophagitis

Appendix 5: Clinical Results

1. Endoscopic findings.

Date……Patient code No…….....

Name……..……

☐ Normal ☐ Gastritis ☐ Gastric ulcer ☐ Duodenitis

☐ Duodenal ulcer ☐ Lymphoma ☐ Adenocarcinoma

2. Histopathology results.

☐ *H. pylori* positive ☐ *H. pylori* negative

A. Assessment of Gastritis

☐ none ☐ mild ☐ moderate ☐ severe

B. Degree of inflammatory activity.

☐ none ☐ 1-2 crypts involved ☐ 25 -50% crypts involved

☐ more than 50% or all cyrpts involved per biopsy.

C. Superficial epithelial damage

☐ None ☐ Slight ☐ Moderate ☐ severe

D. Degree of chronic inflammatory infiltrate in the gastric mucosa.

☐ None ☐ scattered ☐ some areas with dense ☐ nearly to entire mucosa contianing a dense chronic inflammaroty cell infiltrate .

E. Degree of intestinal metaplasia

☐ None ☐ 1 to 4 crypts replaced by intestinal – type epithelium

☐ moderate foci replaced ☐ nearly to entire area of gastric epithelium replaced by intestinal metaplasia.

F. Atrophy

☐ None of gastric glands lost ☐ few and small areas

☐ 25 – 50% gastric glands lost ☐ 50% ir more if gastric glands lost or replaced by intestinal epithelium.

Appendix 6: Distribution of genotypes/Genetic polymorphisms

Date……....… Patients code No ……

Tick the genotypes present

A. VacA polymorphisms

*vac*A s1

*vac*A s2

*vac*A m1

*vac*A m2

*vac*A i1

*vac*A i2

B.CagA polymorphisms,

*cag*A

In the EPIYA motif patterns indicate the number of occurrence

EPIYA motif A

EPIYA motif B

EPIYA motif C

EPIYA motif D

Appendix 7: Abstracts for published journals.

Int J Med Health Sci. Oct 2015, Vol-4; Issue-4 441-447

International Journal of Medical and Health Sciences

Journal Home Page: http://www.ijmhs.net ISSN: 2277-4505

Clinical Relevance of CagA EPIYA Motifs In*Helicobacter pylori* Among the Dyspeptic Patients in Kenya

Lawrence Guantai M'itonga[1*], Andrew Nyerere Kimanga[2], Caroline Wangari Ngugi[2], Thomas M. Mutie[3] [1]Ministry of Education Science and Technology, [2]College of Health Sciences, Jomo Kenyatta University of Agriculture and Technology, [3]Kenyatta National Hospital, Kenya.

ABSTRACT Introduction: The *Helicobacter pylori* cytotoxin-associated gene A (cagA) has been associated with disease severity and its oncogenic potential has been linked to its polymorphic EPIYA motifs whose combinations differ geographically. The aim of this study was to determine the frequency of cagA-positive *Helicobacter pylori* isolated from dyspeptic patients in Kenya and to assess the association of cagA EPIYA motif patterns with clinical outcome. **Materials and Methods:** *H. pylori* positivity was determined by histology and molecular diagnostic method directly from the gastric pathologies. The cagA presence and EPIYA Motif patterns were analyzed by polymerase chain reaction from the *H. pylori* positive samples. **Results:** The cagA gene was identified in 48.75% of the *H. pylori*- patients. The cagA per se was not significantly associated with the gastroduodenal diseases. The most occurring EPIYA pattern was the ABC (56.41%) followed by ABCC at 43% and AB at 28.21%. The presumed virulent ABCCC pattern was rare (5.13%). Increase in the number of EPIYA C (more than one C repeat) repeats was significantly associated gastric cancer (OR=6.577 95% CI 1.620-26.704, p=0.008). **Conclusion:** All the Kenyan cagA EPIYA patterns were of Western type. We found that infection by *H. pylori* cagA strains with multiple EPIYA C repeats to be associated with gastric cancer but not peptic ulcer in Kenya; but the low prevalence of these strains might contribute to the low incidence of gastric cancer in this country. Determining the EPIYA motifs in CagA, rather than detecting cagA gene alone, would be a better marker for assessing the risk of serious gastric pathology.

KEYWORDS: *Helicobacter pylori* cagA genotype, EPIYA Motif, Gastro duodenal diseases

IJHSR.2015; 5(9): 436-444

Association of Helicobacter Pylori VacA Gene Polymorphisms and CagA Gene with Clinical Outcome in Dyspeptic Patients.

Lawrence Guantai M'itonga, Andrew Nyerere Kimanga, Caroline Wangari Ngugi, Thomas M. Mutie.

Abstract

Background: Gastroduodenal diseases have been associated with Helicobacter pylori infection. Cytotoxin-associated gene A (cagA) and different isoforms of vacuolating cytotoxin (vacA) gene have been associated with disease severity. The prevalence and association of these genotypes with severity of disease differ geographically. The aim of this study was to determine the prevalence of the cagA and vacA variants of Helicobacter pylori isolated from dyspeptic patients in Kenya and to assess their association with clinical outcome

Methods: One hundred and twenty seven dyspeptic patients were enrolled into the study. H. pylori positivity was determined by histology and molecular diagnostic method directly from the gastric biopsies collected. The presence of cagA and vacA variants was analyzed by polymerase chain reaction (PCR) from the eighty H. pylori positive samples with various gastroduodenal diseases.

Results: The H. pylori DNA was detected in 62.99% of the dyspeptic patients. The prevalence of cagA gene was 48.75% among the positive samples and was not significantly associated with the gastroduodenal diseases. The less virulent vacA alleles: m2, i2 and s2 were most occurring at 65%, 52% and 49%, respectively. The vacA allele m1, i1, and s1 were significantly associated with peptic ulcer, intestinal metaplasia and gastric cancer, all at $P < 0.05$.

Conclusion: The less toxigenic vacA isoforms s2, m2 and i2 are highly distributed in Kenya. We confirmed the significant association of the vac s1, m1 and i1 with gastric cancer and peptic ulcer. We cannot rule out the possibility of the presence of unidentified strain or certain host or environmental factors that trigger H. pylori-induced cancer and peptic ulceration.

Key words: Helicobacter pylori, vacA gene polymorphisms, Cytotoxin-associated gene A, Clinical outcome

Appendix 8. Ethics and Research Committee Approval

UNIVERSITY OF NAIROBI
COLLEGE OF HEALTH SCIENCES
P O BOX 19676 Code 00202
Telegrams: varsity
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KNH/UON-ERC
Email: uonknh_erc@uonbi.ac.ke
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KENYATTA NATIONAL HOSPITAL APPROVED 08 OCT 2012 & RESEARCH COMMITTEE

Ref: KNH-ERC/A/291

8th October 2012

Lawrence Guantai M'Itonga
Institute of Tropical Medicine and Infectious Disease
JKUAT

Dear Lawrence

Research proposal: Association of Specific *Helicobacter pylori* cagA and vacA gene polymorphisms and clinical outcome in patients with Dyspepsia presenting at Kenyatta National Hospital, Kenya" (P355/06/2012)

This is to inform you that the KNH/UoN-Ethics & Research Committee (KNH/UoN-ERC) has reviewed and **approved** your above revised proposal. The approval periods are 8th October 2012 to 7th October 2013.

This approval is subject to compliance with the following requirements:

a) Only approved documents (informed consents, study instruments, advertising materials etc) will be used.
b) All changes (amendments, deviations, violations etc) are submitted for review and approval by KNH/UoN ERC before implementation.
c) Death and life threatening problems and severe adverse events (SAEs) or unexpected adverse events whether related or unrelated to the study must be reported to the KNH/UoN ERC within 72 hours of notification.
d) Any changes, anticipated or otherwise that may increase the risks or affect safety or welfare of study participants and others or affect the integrity of the research must be reported to KNH/UoN ERC within 72 hours.
e) Submission of a request for renewal of approval at least 60 days prior to expiry of the approval period. (*Attach a comprehensive progress report to support the renewal*).
f) Clearance for export of biological specimens must be obtained from KNH/UoN-Ethics & Research Committee for each batch of shipment.
g) Submission of an *executive summary* report within 90 days upon completion of the study
This information will form part of the data base that will be consulted in future when processing related research studies so as to minimize chances of study duplication and/or plagiarism.

For more details consult the KNH/UoN ERC website **www.uonbi.ac.ke/activities/KNHUoN**

Yours sincerely

PROF. A.N. GUANTAI
SECRETARY, KNH/UON-ERC

c.c. [illegible]