DISSERTATION

DETECTION OF HELICOBACTER PYLORI USING IMMUNOHISTOCHEMISTRY AT THE KENYATTA NATIONAL HOSPITAL HISTOPATHOLOGY LABORATORY

A DISSERTATION SUBMITTED IN PART FULFILLMENT FOR THE AWARD OF DEGREE IN MASTERS OF MEDICINE, HUMAN PATHOLOGY, AT THE UNIVERSITY OF NAIROBI

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DECLARATION

I hereby declare that this dissertation is my original work under the guidance of my supervisors and has not, to the best of my knowledge, been submitted to any university or institution of higher learning as research for award of a degree.

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DEDICATION

To my dear friend Juddy Gachoka; a life well lived.

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LIST OF ABBREVIATIONS

°C	Degrees Celsius
cagA	cytotoxin-associated gene A
CD4	Cluster of Differentiation 4
CD8	Cluster of Differentiation 8
CO_2	Carbon dioxide
DNA	Deoxyribonucleic acid
GERD	Gastro-oesophageal Reflux Disease
H&E	Haematoxylin and Eosin
H. pylori	Helicobacter pylori
HP-NAP	Helicobacter pylori - Neutrophil Activating Protein
IL	Interleukin
KNH	Kenyatta National Hospital
LPS	Lipopolysaccharide
MALT	Mucosal-associated Lymphoid Tumour
Mbp	Maltose binding protein
MIP	Macrophage Inflammatory Protein
NF	Nuclear Factor
O_2	Oxygen
OMP	Outer Membrane Protein
PAI	Pathogenicity Island
PCR	Polymerase Chain Reaction
PPI	Proton Pump Inhibitor
PUD	Peptic Ulcer Disease
ROS	Reactive Oxygen Species
SOD	Superoxide Dismutase
TNF	Tumour Necrosis Factor
UON	University of Nairobi
vacA	vacuolatingcytotoxin A
WHO	World Health Organization
WS	Warthin-Starry

ABSTRACT

Background: *Helicobacter pylori*, is a Gram negative bacteria that colonizes the gastric mucosa and is responsible for a myriad of upper gastrointestinal symptoms. This infection has a higher prevalence in the developing world with prevalence rates estimated to be as high as 80% in some regions. Several tests have been developed to diagnose the disease. Commonly, upper gastrointestinal endoscopy and biopsy are carried out to assess the cause and extent of chronic gastritis. Histochemical stains are used to detect the bacteria. However, these stains have been shown to perform poorly when compared to immunohistochemistry. There is a need to improve on the detection rates so as to properly diagnose the cause of the gastritis to enable clinicians provide appropriate treatment to their patients.

Objectives: The broad objective was to detect *Helicobacterpylori* in gastric mucosa biopsies using immunohistochemical methods. The secondary objectives were to review the histomorphology of gastric biopsies submitted to the Kenyatta National Hospital histopathology laboratory andto compare the histopathologic features of Giemsa-negative *H. pylori* biopsies with immunohistochemistry.

Design: This was a cross-sectional descriptive study carried out from March 2016 to May 2017. Thirty (30) samples were collected prospectively from August 2016 to May 2017.

Setting: The Kenyatta National Hospital Histopathology laboratory in conjunction with the Immunohistochemistry section of the department of Human Pathology, University of Nairobi.

Materials and methods: This was a descriptive cross-sectional study. Thirty samples were collected from the Endoscopy Unit of Kenyatta National Hospital and forty-eight stored samples in the Histopathology laboratory were retrieved. Haematoxylin and Eosin and Giemsa staining was routinely carried out to describe the pattern of gastritis and presence of *Helicobacter pylori* infection. Samples that turned out negative for infection underwent immunohistochemical staining to detect *Helicobacter pylori*. Data collected using a modified tool based on the Updated Sydney classification of gastritis andwas analyzed using SPSS version 21.0. Assessment was done to correlate if there was a significant improvement in the detection rates based on the various histopathologic findings seen on routine staining.

Results: A total of eighty-nine (89) gastric biopsies were selected for review, of which 11 samples were found to have been positive for *H. pylori* on repeat Giemsa staining. They were therefore excluded resulting in a sample size of 78 for immunohistochemistry staining. The mean

age of the patient was 48.9 years with a standard deviation of 18.7. The Male-to-Female ratio was 1:1.1.Chronic inactive gastritis was the most common Hematoxylin and Eosin finding, which was seen in 82.1% of the cases. Severe inflammation (+3) was present in half (50%) of the samples. Positivity was found in 25.6% (20 of 78) of the samples. There was low bacterial colonization in 85% (17 of 20) of cases. Medium quality of organism staining was present in 70% of the positive cases while background staining had medium quality in 83.3% of the samples.Presence of lymphoid aggregates correlated significantly with positive staining (p= 0.032, OR 3.1). No other histopathologic finding correlated significantly with immunohistochemistry positivity.

Conclusions: Immunohistochemistry is a reliable technique in detection of *Helicobacter pylori*. It is superior to Giemsa stain for detecting *H. pylori* infection, especially when lymphoid aggregates are present. Hematoxylin and eosin stain adequately displays the inflammatory and adaptive changes associated with *H. pylori* infection. Giemsa staining still remains the preferred technique to visualize *H .pylori* in gastric biopsies.

1.0 INTRODUCTION

The term “gastritis” was first used in 1728 by the German physician, Georg Ernst Stahl to describe the inflammation of the inner lining of the stomach. In the past many considered gastritis a useful histological finding, but not a disease(1). This all changed with the discovery of *Helicobacterpylori* by Robin Warren and Barry Marshall in 1982 leading to the identification, description and classification of a multitude of different gastritides(2).

Helicobacterpylori (H. pylori) is a spiral-shaped Gram-negative rod that colonizes the gastric mucosa.It has colonized humans naturally for over 50,000 years. It is the main risk factor for antral gastritis, peptic ulcers, gastric ulcers, gastric adenocarcinoma and gastric mucosa-associated lymphoid tumor (MALT)(3)

In this study, the histopathology findings on Haematoxylin and Giemsa staining are correlated with immunohistochemistry. Improved detection of the bacteria will aid clinicians provide the correct treatment to their patients.

1.1 EPIDEMIOLOGY OF H. PYLORI

Infection with *H. pylori* causes what is perhaps the most prevalent disease in the world(4).It is mostly acquired in childhood and by the age of 10years; more than 50% of children worldwide carry the organism. A declining prevalence in developed countries may be due to decreased transmission because of less crowding and frequent exposure to antimicrobials. It is estimated that 80% of the population in developing parts of the world are infected by age 20(3).

In Kenya, a study done in 696 patients with dyspepsia revealed the prevalence among children at 73.3% and among adults at 54.8%. Infection rates were 56% in rural Kenyan Africans, 62% in urban Kenyan Africans and in urban Kenyan Asians at 58%. This was determined by endoscopic evaluation.(5)

In another study, 445 stool antigen tests for children between 18months and 15years old were performed. *H.pylori* was positive in 99 (22%) children; with 64% of the positive tests from children aged 8years and above. In the same period, *H.pylori* was identified in 44 out of 74 (59%)endoscopy biopsies with the youngest patient aged 3years. In all cases, there was equal distribution among males and females.(6)

In his dissertation, Dr. Solomon Sava detected a prevalence of 50.3% among 175 children; of

whom 64.8% had positivity for cag-A in their serum(7). Anotherdissertation by Dr. Peter Ochung'o detected *H. pylori* in 24 of 78 (30.5%)tonsillar tissues with chronic recurrent tonsillitis and adenotonsillar hypertrophy in children with obstructive sleep apnea syndrome. (8)
The risk factors for acquisition for infection include low socioeconomic status, birth or residence in a developing country, domestic crowding, unsanitary living conditions, unclean food or water and exposure to gastric contents of an infected individual(3)

1.2 MICROBIOLOGY OF H.PYLORI

1.2.1Morphology

H. pylori is Gram negative, motile, 2-4μm in length and 0.5-1μm in width. It is usually spiral-shaped, though may appear as a rod. Coccoid forms are present in extra-gastric sites such as tonsillar crypts and are thought to be reservoirs of infection.(9)They also appear after antibiotic therapy or prolonged culture. It has 2 to 6 unipolar, sheathed flagella approximately 3μm in length. It lacks fimbrialadhesins.

The cell envelope is similar to other Gram negative bacteria. The outer membrane is comprised of phospholipids and lipopolysaccharide (LPS). The phospholipids contain cholesterol glucosides; a rarity in bacteria. The LPS consists of lipid A, core oligosaccharide and O side chain.

1.2.2Growth Requirements

H. pylori is microaerophilic; with optimal growth at 2-5% O_2 levels, 5-10% CO_2 levels, high humidity, 34-40°C (though optimal growth is at 37°C), pH 5.5 to 8.0 (with optimal growth at neutral pH). It is fastidious and requires complex growth media such as Dent or Skirrow's media, supplemented with blood or serum. Isolation from gastric biopsies is often difficult.

Inspection of cultures should be done from days 3-14. Colonies are 1mm in diameter, translucent and smooth. Optical detection can be enhanced by supplementing with triphenyltetrazolium chloride (TTC), which is reduced to deep red formazon, causing colonies to appear dark red.

1.2.3Metabolism

H.pylori is catalase-, urease- and oxidase positive. It also metabolizes glucose. It lacks biosynthetic pathways for several amino acids.

i. Respiratory and oxidative stress defence

H. pylori utilizes oxygen as a terminal electron receptor to combat oxidative stress from the immune response via SOD and catalase. The neutrophil activating protein (HP-NAP) protects DNA from effects of reactive oxygen species.

ii. Nitrogen metabolism

Nitrogen is acquired from amino acids and urea in the gastric mucosa. *H. pylori* can use various pathways in ammonia synthesis depending on the environmental conditions.

The main route is through urease, which makes up 10% of the total protein content. It is required for amino acid synthesis, acid resistance and virulence. Urea enters the cell via the H^+-gated urea gated channel, UreI, resulting in increased transport in acidic conditions.

iii. Metal metabolism

Metals are essential as enzyme cofactors, maintaining osmotic pressure and catalyzing electron transport, redox reactions and energy generation. They include nickel, iron, copper and cobalt.(10)

1.2.4Virulence Factors

1. cagA (cytotoxin-associated gene A)

Most patients infected with *H.pylori* usually have few clinical symptoms despite having chronic active gastritis. This is because some strains are less virulent than others, due to differences in cagA positivity. cagA is a highly immunogenic and results in a more significant inflammatory response. It encodes proteins that are responsible for penetrating gastric epithelial cells and induces apoptosis of T cells. This results in epithelial cell morphologic changes that facilitate lifelong colonization.

2. vacA (vacuolatingcytotoxinA)

vacA forms pores in epithelial membranes, releasing urea and anions. It also enters the cytoplasm and induces mitochondrial apoptosis. It disrupts function of B-cells, CD8+ Tcells, macrophages and mast cells, inhibitsantigen presentation and CD4+ T-cell proliferation. vacA is linked to $cagA^+$ genotype resulting in higher virulence.

3. Adhesins and OMPs
    a. BabA (HopS) - binds to fucosylated Lewis b antigens on host cells.
    b. SabA - binds to sialyl-Le and activates neutrophils in a non-opsonic manner. It assists in transfer of other virulence factors.

c. Oip (HopH)- associated with IL-8 induction and increases risk of gastric cancer.

4. LPS

It stimulates production of IL-8 and NF-κβ in epithelial cells and immune cells. It is also involved in molecular mimicry; contributing to persistent infection. In some subjects, this mimicry has led to inducing autoantibodies against the proton pump of parietal cells.

5. Acid resistance

Urease converts urea to ammonia and bicarbonate. Ammonia is cytotoxic to gastric epithelial cells, bicarbonate suppresses the bactericidal effect of peroxynitrite; a nitric oxide metabolite. The urease subunits UreA and UreB are immunologically active proteins.(10)

1.3PATHOGENESIS AND PATHOLOGY

H.pylori grows optimally at pH 6.0-7.0. Gastric mucous is impermeable to acid and has a strong buffering capacity. *H.pylori* is found deep in the mucous layer near the epithelial surface where physiological pH is present. *H.pylori* is motile and can move from the mucous layer to the epithelial surface resulting in mucosal damage and inflammation.(11)Colonizationby *H. pylori*induces a specific tissue response - chronic superficial gastritis - which is accompanied by cell-mediated and humoral responses. There also is down regulation of the immune system resulting in ineffective clearance of the bacteria. Development of overt disease depends on a complex interplay between bacterial strain differences, host susceptibility to disease and environmental factors.(3)

Acute infection causes marked inflammation in the antrum and body, inhibition of parietal cell function and eventually hypochlorhydria. There is increased gastrin level due to lack of normal inhibition of gastrin release exerted by gastric acid and loss of G cells with antral mucosa atrophy. Functional inhibition of gastric acid secretion occurs by: production of IL-1β; which is the most important inhibitor of acid secretion yet identified and ross-reaction of antibodies with the proton pump of the parietal cell.(12)

Persistent infection leads to atrophy with increased risk of adenocarcinoma; which develops due to epithelial cell proliferation in a setting of chronic inflammation. Certain strains contain a pathogenicity island that has cytotoxin-associated A (cag-A) gene which penetrates epithelial cells. This initiates a signal cascade akin to unregulated growth factor stimulation.

Malignancy tends to occur against a background of a body predominant gastritis and presence of atrophy. This may be related to the production of potentially carcinogenic nitrosoamines from bacteria which colonize the gastric lumen at pH above 4. There is also increased gastric juice nitrite levels with reduced ascorbic acid; hence increased nitrosoamine levels.(12)

Gastric mucosa-associated lymphoid tumors develop when T-cells, in the setting of pro-inflammatory cytokines such as IL-1β and TNF, stimulate polyclonal B-cell proliferation. In this setting activation of transcription factor NF-κβ is dependent on T-cell activity. Hence *H.pylori* eradication results in cure of the MALT-omas that develop. However, in later stages, additional mutations results in NF-κβ activation independent of T-cell stimulation. The MALT-omawill then have the capacity to metastasize.(13)

In duodenal ulcer disease, *H.pylori* reduces bicarbonate secretion causing mucosal damage and gastric metaplasia. This enables *H. pylori* to colonize this region. There is also elevated gastrin levels.(12)

1.4IMMUNE RESPONSE TO INFECTION

Infection by*H.pylori* induces pathology that is driven by Th1 cells and Th1 cytokines that recruit mononuclear cells that eliminate the bacteria.(14)

H.pylori always induces a strong immune response but with ineffective clearance of infection by down-regulating inflammation. IL-2, which is produced by mononuclear cells, causes differentiation of naïve T cells into Th1 cells.(10)Toll-like receptors on epithelial cells recognize and react to flagella, peptidoglycan and LPS. There is resulting NF-κβ activation and chemokine expression on the gastric epithelial cells. However, not all bacteria attach to the epithelial cells; which influences the degree of the resultant inflammation.

H.pylori infection causes up-regulation of MIP-3α gene in gastric epithelial cells. However, macrophage phagocytosis is inhibited; in an unknown pathway. Lewis blood group antigens on LPS block Th1 cell activation and IL-6 production. The Lewis antigen also undergoes phase variation which aids in immune evasion.IL-1β is a potent pro-inflammatory cytokine. It is also the most potent inhibitor of acid secretion. This results in corpus-predominant colonization, pangastritis, atrophy and increased risk of malignancy.TNF-α is a pro-inflammatory cytokine which influences gastrin production. IL-10 is an anti-inflammatory cytokine associated with increased risk of malignancy when levels are reduced.(14)

1.5 CLINICAL ASPECTS OF H.PYLORI-ASSOCIATED DISEASES

i. Acute gastritis

The acute phase of colonization is associated with transient non-specific dyspeptic symptoms with inflammation of the proximal and distal mucosa, or pangastritis. It is unclear if spontaneous clearance and resolution of gastritis occurs and, if so, how often.(10)

ii. Chronic gastritis

In subjects with intact acid secretion, colonization is predominantly antral, where few parietal cells are present. Histopathologic evaluation reveals limited chronic active inflammation with low numbers of bacteria. Where acid secretion is impaired, bacteria are evenly distributed with closer mucosal contact in the corpus, resulting in corpus-predominant pangastritis. Reduced acid secretion can occur due to gastric atrophy, vagotomy or acid-suppressive drugs, in particular PPIs.

Active inflammation of the corpus augments hypochlorhydria, with local inflammatory factors such as interleukin-1β (IL-1β) suppressing parietal cell function.

iii. Peptic ulcer disease

Ulcers are mucosal defects more than 5mm in diameter and penetrate the muscularis mucosa. Ulceration occurs where inflammation is most severe. In hypochlorhydria, this is usually at the transition zone between the corpus and antrum. In individuals with normal to high acid output, inflammation is most severe at the distal stomach and proximal duodenum. Gastric ulcers commonly occur along the lesser curvature at the transition from the corpus to antral mucosa. Duodenal ulcers usually appear at the duodenal bulb.

Duodenal ulcers predominantly arise between ages 20 and 50 years while gastric ulcers particularly occur above 40years of age and are more common in developing countries. In developed countries, approximately 85% of gastric ulcers and 95% of duodenal ulcers are associated with *H. pylori* infection. Lifetime risk for PUD is 3-10 times higher in *H. pylori* - positive than negative subjects. Ten to fifteen percent of infected individuals eventually develop ulcer disease on long-term followup. However it is unknown if similar disease risks are present in indivduals in developing countries. (10)

H. pylori eradication changes the natural course of PUD and prevents recurrence; which may occur in the setting of renewed infection, NSAID use or idiopathic ulcer disease. Ulcer complications include bleeding, stricture formation and perforation.Bleeding is estimated to

occur in 15-20% of ulcers. A bleeding ulcer is the cause of upper gastrointestinal bleeding in approximately 40% of patients. The primary treatment is endoscopic therapy. Eradication of *H. pylori* reduces the risk of renewed bleeding.(15)

iv. Atrophic gastritis, intestinal metaplasia and gastric cancer

In approximately half of *H. pylori* infected subjects, they developed atrophic gastritis and intestinal metaplasia. This occurs where inflammation is most severe and increases the risk for gastric cancer by 5- to 90- fold. Bacterial and host factors influence the severity of the chronic inflammatory response.

Gastric cancer is the fourth most common cancer worldwide. *H. pylori* was designated a class I carcinogen by WHO, as it increased risk by up to 10-fold. In Western countries, the lifetime risk of gastric cancer is estimated at 1-2% of individuals infected with *H. pylori*linked to 60-80% of the cases.

Several randomized studies report that eradication of *H. pylori* can halt progression, or to some extent, induce a regression of atrophy. This effect is less evident in gastric cancer prevention. Regression of gastric atrophy is however of no direct relevance to cancer risk. Therefore, prevention of cancer is a major research topic on *H. pylori*.(15)

v. Gastric MALT lymphoma

MALT appears in response to *H. pylori* colonization where a monoclonal population of B cells slowly proliferate. MALTomas occur in less than 1% of *H.pylori* positive subjects. Complete remission in stage 1E lymphoma confined to the stomach occurs in 60-80% of subjects following *H. pylori* eradication. However, 10-35% who reach remission show recurrent disease. A major predictor of response is the t(11;18),(q21,q21) translocation associated with AP12-MALT1 fusion. The fusion suppresses apoptosis therefore the MALTomas rarely respond to *H. pylori* eradication.(13), (10)

vi. Gastroesophageal reflux disease

H. pylori infection may protect against GERD due to acid-suppressive effect of inflammation of the corpus. However, eradication has no impact on new development or worsening of pre-existing GERD.

vii. Extragastroduodenal disorders

H.pylori has been linked to coronary heart disease, dermatologic disorders, thrombocytopenic purpura, iron deficiency anemia and Guillain-Barré syndrome. It is also associated with chronic recurrent tonsillitis.(8)The hypothetical mechanisms include chronic activation of the coagulation cascade, antigenic mimicry and accelerating artherosclerosis. Eradication of *H. pylori* leads to improved thrombocyte counts in patients with idiopathic thrombocytopenic purpura. However, in the other conditions, no improvement was noted with eradication.(10)

The diagram below summarizes the natural history of *H. pylori* infection(16) (as explained previously)

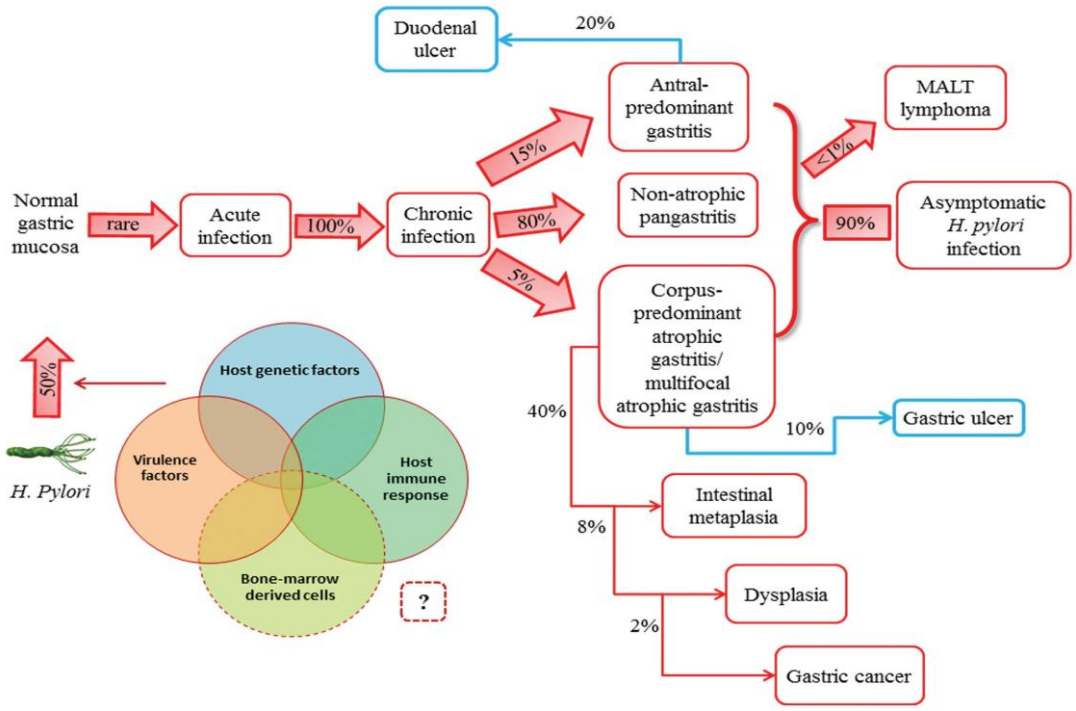


Figure 1: The natural history of Helicobacter pylori infection.
(Adapted from Conteducaetal.*H. pylori* infection and gastric cancer: State of the art review,International Journal of Oncology 42: 5-18, 2013)

1.6DIAGNOSIS OF H. PYLORI INFECTION

The available tests are divided into invasive tests; based on gastric specimens, and noninvasive tests; based on peripheral samples. The choice of the test depends on the clinical setting and on local experience.The tests have different sensitvity and specificity due to use of different kits from various manufacturers. However, histological detection is still taken as the gold standard test for diagnosing infection.(17)For routine diagnosis, histologyand culture, urea breath test and

stool antigen test are used most often while serology is used mainly in epidemiological studies.(10)Upper gastrointestinal endoscopy is used to assess the effect of infection. Assessment of gastric biopsies enables the histopathologist determine the cause of the gastritis through routine staining.

The table below describes the advantages and disadvantages of the tests most commonly used.(3)

TEST	ADVANTAGES	DISADVANTAGES
Invasive (based on endoscopic biopsy)		
Urease test	Quick, simple	Some kits are not sensitive before 24hours
Histology	Provides additional information	Depends on user experience and use of histochemical stains
Culture	Tests antibiotic sensitivity	Dependent on user experience
Non-invasive		
Serology	Inexpensive, convenient Affected less by antibiotic or PPI use	Not used for follow up Less accurate than breath test Some kits are inaccurate
^{13}C urea breath test	Inexpensive Simpler than endoscopy Ideal for follow up	Requires a period of fasting Less convenient
Stool antigen test	Inexpensive, convenient Useful for follow up Useful in children	Less accurate than breath tests; especially for follow up

Table 1: Advantages and disadvantages of diagnostic tests.
(Adapted from Harrison's Principles of Internal Medicine (2012), 18th Edition)

1.6.1 The histology of the stomach

The layers of the stomach include the mucosa, submucosa, muscularis mucosa and serosa. It consists of three histologically distinct regions: cardia, fundus/main body (corpus) and pylorus. The cardia is adjacent to the esophagus and contains mucus glands that lubricate the entrance of the stomach. It has pits and glands, which are of equal length. The fundus contains shallow pits and elongated glands with darkly stained clusters of cells in deep regions. The pits are lined by simple columnar cells that secrete mucus. The glands are composed of parietal cells that secrete hydrogen, chloride, bicarbonate ions and intrinsic factor. The pyloric region consists mainly of mucus cells with pits occupying a greater portion of the mucosa.(18)

1.6.2 Histopathologic findings in H. pylori gastritis

1.6.2.1Acute gastritis

The early phase of infection elicits an acute inflammatory response with short-lived clinical manifestations or none at all. Therefore, pathological aspects are limited to a few well-documented case reports. On endoscopy, hemorrhagic lesions and erosions or ulcers, mainly in the antrum are encountered.(19)

1.6.2.2 Chronic gastritis

In histopathologic sections, *H. pylori* is most commonly found in the antrum. It may exist in the mucus, surface or intracellularly with greater epithelial damage in intracellular colonization.(17) However, intracellular invasion has been observed especially in patients on PPIs.(19)It causes disintegration and apical mucus loss with formation of epithelial pits. There is inflammatory cell infiltrate predominated by neutrophils from the lamina propria to the epithelial surface.(17)

Various histopathological features are seen in the mucosa, such as:

i. Neutrophil infiltration - the acute phase of infection reveals moderate to severe infiltration. The neutrophils may fill the lumen of the gastric pits, forming microabscesses and surface exudates.
ii. Mononuclear infiltration- mucosal infiltrates with lymphocytes, plasma cells and mast cells is characteristic of chronicity. They may persist long after eradication.
iii. Lymphoid aggregates and follicles - these are detected in virtually all subjects with *H.pylori* gastritis. In children and young adults, the follicles lead to the endoscopic appearance of nodularity; often referred to as follicular gastritis.
iv. Eosinophil infiltration- in adults, this may be a mild part of the entire inflammatory infiltrate. However, in children it is marked and may persist after eradication of the infection.
v. Mucosal hyperaemia and edema - this is caused by an increase in mast cells.
vi. Surface epithelium degeneration - this occurs due to epithelial injury and necrosis which results in appearance of cuboidal-shaped, rather than columnar-shaped, cells with mucin depletion. Regeneration results in accumulation of buds of cells at the mucosal surface.
vii. Surface erosion - this is associated with a hyperplastic, regenerative epithelium and a superficial layer of fibrinoid necrosis containing neutrophils and cellular debris.

viii. Foveolar (pit) hyperplasia - it is defined as elongation and tortuosity of gastric pits due to cellular proliferation. It is a compensatory tissue response to exfoliation of the epithelium. A mild degree of hyperplasia is present in infection.

ix. Intestinal metaplasia- it is defined as replacement of gastric mucinous epithelial cells with small intestinal (goblet, enterocyte) cells.It is thought that metaplasia is a host defence adaptation to *H. pylori* infection. There is a positive correlation between the degree of metaplasia and risk of progression to carcinoma.

x. Gastric atrophy- this is loss of gastric glands with loss of functional epithelium. This mainly occurs in the antrum. There are two types of atrophy; metaplastic and non-metaplastic. Non-metaplastic atrophy is uncommon, but it is frequently associated with severe atrophic gastritis; which predisposes to gastric cancer.

On examination of histopathology sections, five major patterns of gastritis have been described:

1) Nonatrophicantral predominant gastritis

This is the most common in the Western world. It is associated with normal or increased acid secretion and a 20% risk of duodenal ulcers. It is characterized by:

- Moderate or severe antral inflammation
- Normal or mild inflammation of the corpus
- Lack of atrophy

2) Nonatrophic corpus-predominant gastritis

This is common in patients on long-term PPI use. *H.pylori* density and inflammation are low in the antrum and high in the corpus.

3) Nonatrophicpangastritis

Minimal difference in the intensity of inflammation between the corpus and the antrum is thought to be the background in which atrophy develops. This is common in poorly sanitized areas where *H.pylori* is highly endemic.(19)

4) Antrum-restricted atrophic gastritis

This is characterized by extensive atrophic metaplastic changes with:

- Moderate to severe antral inflammation, and
- Normal or mildly inflamed corpus, without atrophy

5) Multifocal atrophic gastritis

This pattern is common in populations living in suboptimal sanitary conditions. There is severe inflammation in the corpus with reduced acid secretion. It is a risk factor for gastric ulceration,

dysplasia and intestinal-type adenocarcinoma.

1.6.3 The Updated Sydney System

In 1990, based on new etiological facts on gastritis, a new classification system was presented at the World Congress of Gastroenterology in Sydney, Australia. It was later updated in 1994 at the H.pylori congress in Houston, United States. The Sydney system for the classification of gastritis emphasized the importance of combining topographical, morphological and etiological information that would help generate reproducible and clinically useful diagnoses.(20)The report generated includes:

a. The type of gastritis - active, chronic or other.
b. Grade of the presence of *Helicobacter* density, activity (neutrophilic infiltration), chronic inflammation, glandular atrophy, intestinal metaplasia. The grade assigned is +1 to +3 (see Figure 2)
c. Location of gastritis – antrum, fundus/body, cardia, diffuse.
d. Other features (ungraded) such as granulomas, eosinophils, intraepithelial lymphocytes.(19)

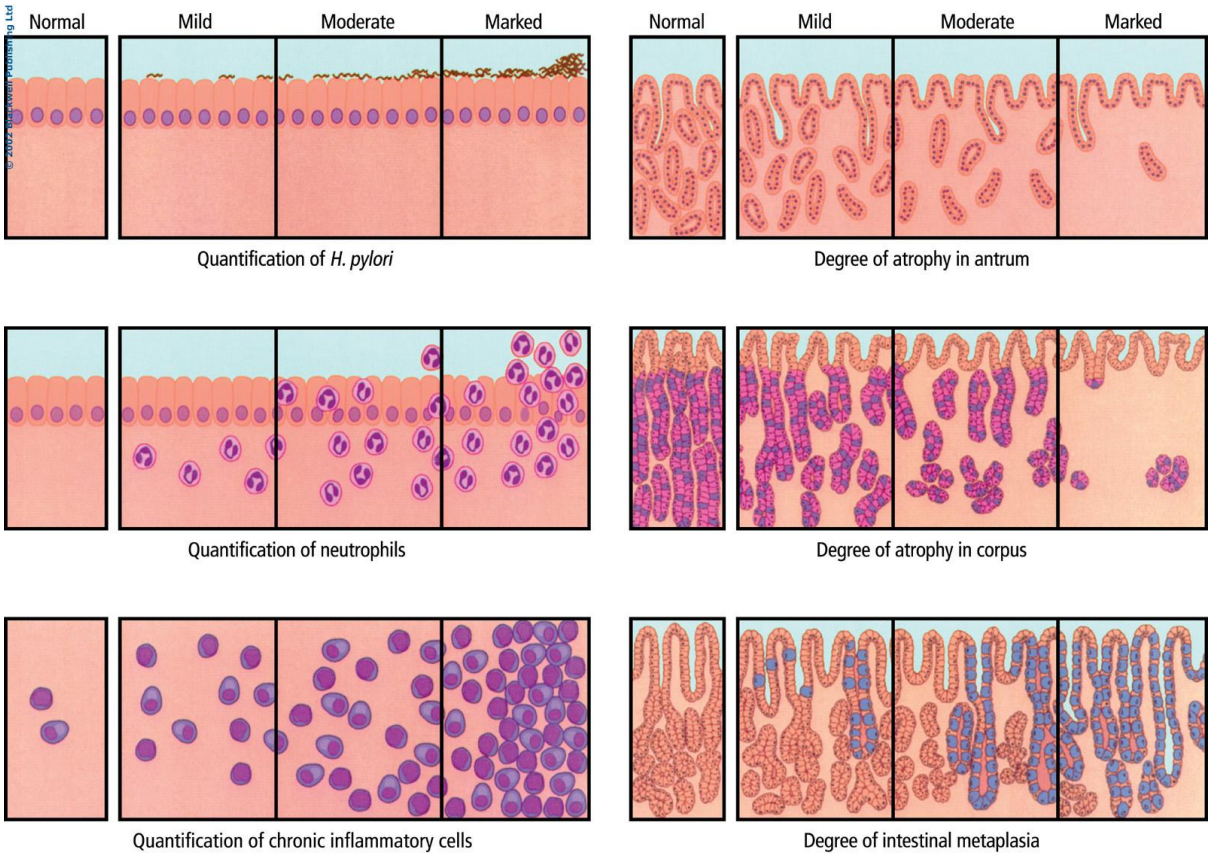


Figure 2: Schematic illustration of the grading of features seen in gastritis in the Updated Sydney Classification of Gastritis.**(21)**

However, in routine practice, few biopsy specimen are submitted; often from unspecified sites. In such cases, no attempt should be made to use the Sydney system. Instead, a less specific diagnosis may be used.(19)

H. pylori can be identified on H&E stain but recognition is enhanced with Giemsa stain. Other histochemical stains enhance detection such asWarthin-Starry or Steiner silver stains,Genta stain and Alcian yellow-toluidine blue method. Recently, newer methods have been introduced such as immunohistochemistry (IHC) and polymerase chain reaction (PCR). PCR has improved detection by 20-40% in histologically negative biopsies.(17)

2.0 LITERATURE REVIEW

2.1IMMUNOHISTOCHEMICAL DIAGNOSIS OF HELICOBACTER PYLORI

Immunohistochemistry combines histological, immunological and biochemical techniques for identifying cellular or tissue antigens by means of antigen-antibody interactions. The site of antibody binding is identified by either direct labeling antibody or using a secondary labeling method. The visual marker may be a fluorescent dye, colloidal metal, hapten, radioactive marker or enzymatic marker for light microscopy. Background or non-specific staining is minimized to highlight the antigenic reactivity.

The principle has existed since the 1930s. The use of avidin-biotin complex was developed in the early 1980s. Both monoclonal and polyclonal antibodies have been developed.

Current applications of IHC include:

1. Analysis of tumors of uncertain origin such as metastases and poorly differentiated tumors.
2. Predicting response of therapy, for example ER/PR receptor status in breast malignancy.
3. Diagnosis of infectious diseases whereby rapid results can be obtained for agents that may be difficult to grow or require long incubation such as mycobacteria, fungi and viruses.
4. Diagnosis of degenerative brain disease and muscular dystrophies.(22)

Detection of *H. pylori* using immunohistochemical methods has been done for several years. In most studies, IHC was used as the gold standard method to detect *H.pylori* in gastric biopsies. Among the earliest studies done, IHC was compared to H&E, Giemsa and Warthin-Starry stains. IHC had higher detection rates (66% as compared to 61% on WS, 55% on Giemsa, 37% on H&E). The researchersconcluded that IHC is easy to use and highly specific in detecting *H. pylori* in gastric biopsy and resection specimens.(23)

A recent study conducted to compare H&E, Giemsa and toluidine blue staining with IHC reviewed 54 gastric biopsy specimens. *H. pylori* was positively identified by IHC in 43 (79.63%) patients, 18 (33.33%) by H&E, 24 (44.44%) by Giemsa and 33 (61.11%) using toluidine blue staining methods.(24)

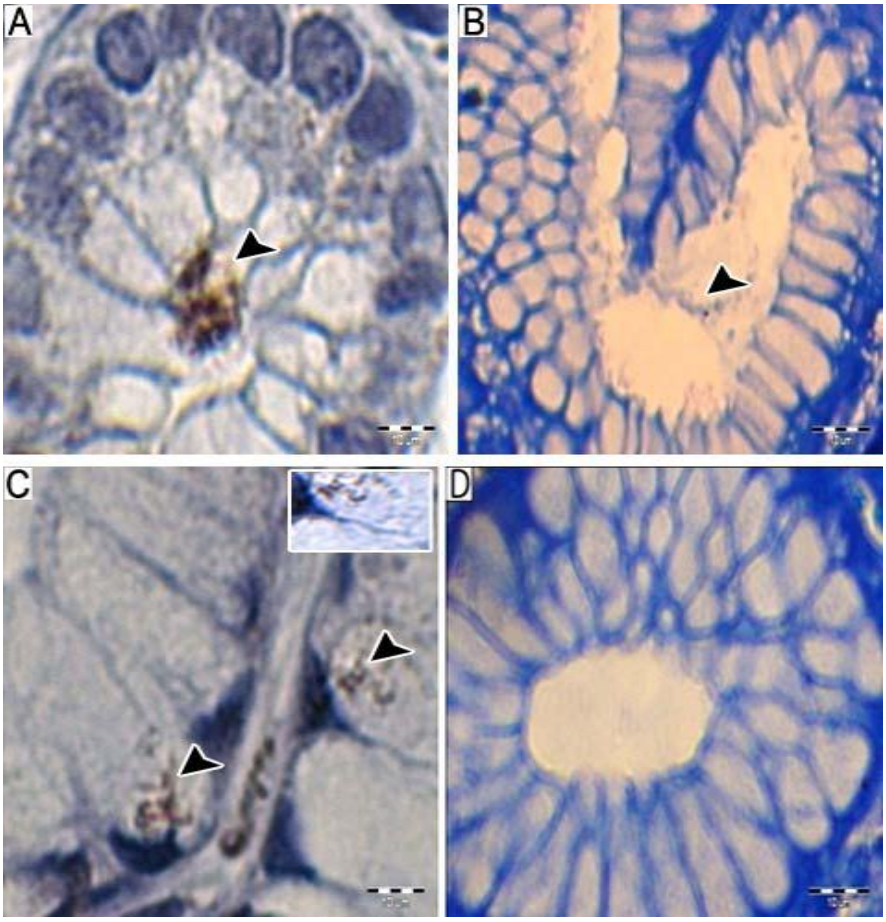


Figure 3: Comparative detection of *H. pylori* by IHC and histochemical staining.

(A) The obvious identification of cluster of modified coccoid forms of *Helicobacter pylori* by immunohistochemistry (arrowhead)

(B) coccoid*H. pylori*onGiemsa stained section (arrowhead)

(C) IHC stain for *H. pylori* shows a small area with organisms inside the epithelial cells. Inset shows individual *H. pylori* with characteristic elongated, slightly spiral S shaped (arrowheads)

(D) Giemsa stained biopsy of the same sample as (C) showing absence of the organisms(24)

(Adapted from Tajallietal.The Immunohistochemistry and Toluidine Blue Roles for *Helicobacter pylori* Detection in Patients with Gastritis.*Iranian Biomedical Journal 17* (1): p36-41 (January 2013)

The table belowsummarizes the findings of the study according to the Updated Sydney System vis-à-vis the staining methods used. It is evident that where there is mild gastritis, H&E and Giemsa stains had poor *H. pylori* detection rates whereas IHC had much higher detection rates.

Table 2.Detection of *Helicobacter pylori* using H&E, Giemsa, toluidine blue and IHC

Degree of inflammation (no. of cases)	H&E		Giemsa		Toluidine blue		IHC	
	+No. (%)	-No. (%)	+No. (%)	-No. (%)	+No. (%)	-No. (%)	+No. (%)	-No. (%)
Mild chronic inactive gastritis (24)	1(4.2)	23 (95.8)	5 (20.8)	19 (79.2)	9 (37.5)	15 (62.5)	15 (62.5)	9 (37.5)
Moderate chronic inactive gastritis (5)	2 (40.0)	3 (60.0)	3 (60.0)	2 (40.0)	3 (60.0)	2 (40.0)	4 (80.0)	1 (20.0)
Mild chronic active gastritis (9)	2 (22.2)	7 (77.8)	2 (22.2)	7 (77.8)	6 (66.6)	3 (33.3)	8 (88.8)	1 (11.1)
Moderate chronic active gastritis (16)	13 (81.25)	3 (18.75)	14 (87.5)	2 (22.2)	15 (93.5)	1 (6.25)	16 (100.0)	0 (0)
Total (54)	18 (33.3)	36 (66.7)	24 (44.4)	30 (55.6)	33 (61.1)	21 (38.9)	43 (79.3)	11 (20.1)

staining methods in correlation with degree of inflammation and activity.(24)
(Adapted from Tajallietal.The Immunohistochemistry and Toluidine Blue Roles for *Helicobacter pylori* Detection in Patients with Gastritis.*Iranian Biomedical Journal*)

In a similar study, 79 cases were reviewed. On routine and histochemical stains, presence of *H. pylori* was shownin 26 (32.9%) cases; whereas 49 (62.0%) cases demonstrated *H. pylori* on IHC stain. Immunostains were negative in six cases where *H. pylori* was suspected by routine methods.(22)

However, a study done in Nigeria showed good agreement between Giemsa and IHC. Thirty-five biopsies having a histological diagnosis of chronic gastritis were reviewed. Giemsa showed a sensitivity of 85.7%; specificity of 92.9%; accuracy of 88.6%. Its positive predictive value was 94.7% and negative predictive value of 81.3%. The abstract however did not explain the methods used and the results for IHC weren't shown. A sample size of thirty-five is also relatively small compared to other studies done.(25)

Most immunohistochemical stains use antibodies that are polyclonal in nature. Newer monoclonal antibodies have been developed. One report scored the quality of organism morphology and background from +1 to +3. It showed that monoclonal antibody had a 75.6% of cases with high quality organism morphology compared to only 34.4% with polyclonal antibody. On background staining, monoclonal antibody had 95.8% high quality compared with 87.3% with polyclonal antibody.(26)

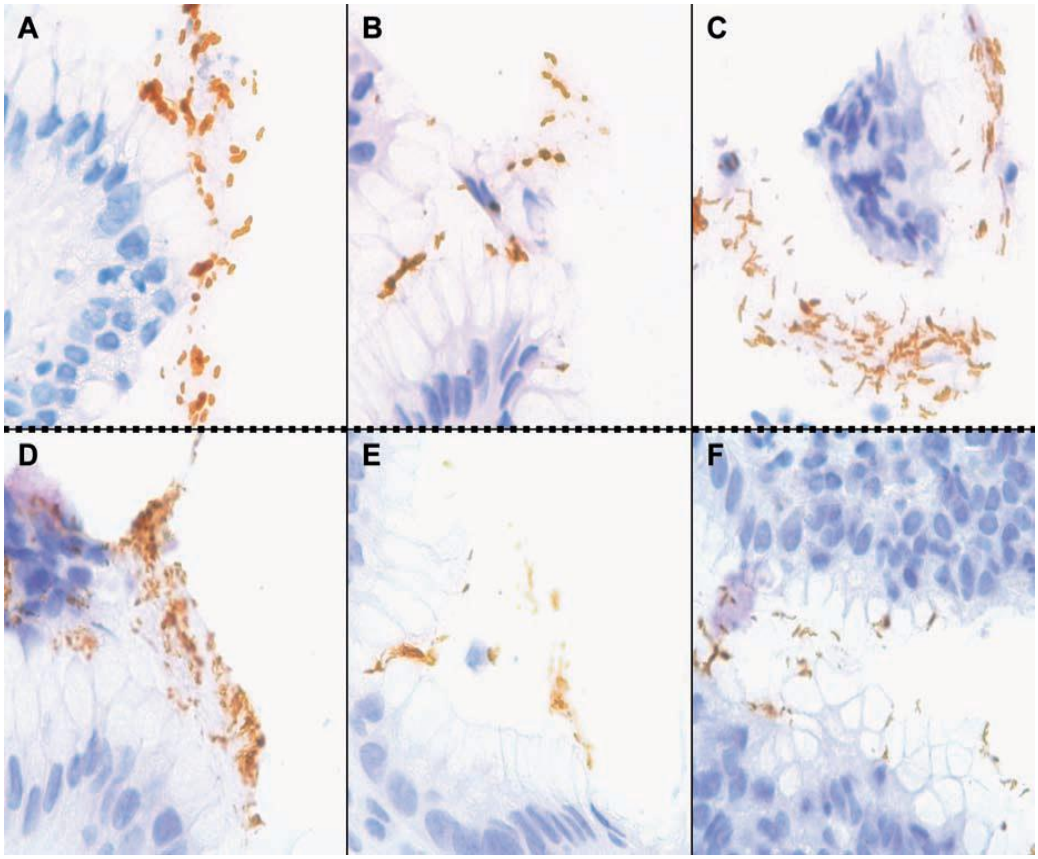


Figure 4: Immunohistochemical images of the monoclonal versus the polyclonal antibodies.

(**A**), (**B**), (**C**): Staining of *H. pylori* monoclonal antibody clone ULC3R on cases 16, 184, and 60, respectively showing superior staining characteristics of the organisms and the background.

(**D**), (**E**), (**F**): Staining of *H. pylori* polyclonal antibody on cases 16, 184, and 60, respectively.(26)

(Adapted from Riba et al. Improved Histologic Identification of *Helicobacterpylori* by Immunohistochemistry using a New Novocastra Monoclonal Antibody.*Labmedicine*,)

The advantages of using H&E to detect *H. pylori* are: it is a well tested method, inexpensive, requires a short time to perform, has relatively highly reproducible results and that it can be used in assessment of morphologic changes. Its disadvantages are: low sensitivity and masking of bacteria by inspissated mucus. The advantages of Giemsa are: it is sensitive, cheap, easy to perform and reproducible while its disadvantages include non-specific staining of mucus, debris or water bath contaminants and poor staining of bacteria closely approximated to the epithelial surface. The advantage of the Warthin-Starry silver stain is that it is the most sensitive histochemical stain. The disadvantages of WS are: it is technically difficult to perform, often not reproducible, non-specific staining for *H. pylori* and it requires high magnification for identifying organisms. The advantages of IHC include: it is less demanding than WS silver stains, reliable,

easy to use, easy to interpret and it is able to detect low numbers of organisms and coccoid forms.The disadvantages of IHC are: financial constraints limit its use for routine purposes, time consuming and negative control needs to be used with every slide.

Recommendations for IHC are when gastric biopsy specimens with chronic gastritis and are negative for *H. pylori* using H&E and Giemsa, for post-treatment biopsy specimens for MALT lymphoma and where coccoid bacteria or other organisms are seen on Giemsa. (23)

3.0 STUDY JUSTIFICATION

There is a high prevalence of *Helicobacterpylori* in the general population. In 2014, of the 208 gastric biopsies submitted to KNH histopathology lab, only 108 cases were positive for *H.pylori* on Giemsa stain. This represents 51.92% of the submitted biopsies. The low detection rates may be due to poor sensitivity of the H&E and Giemsa stains. Immunohistochemistry is a superior technique in determining the cause of chronic gastritis; especially without obvious evidence of *H.pylori*.

Accurate detection of *H.pylori* will assist clinicians provide appropriate treatment to their patients.

3.1 RESEARCH QUESTION

What is the role of immunohistochemistry in detecting *Helicobacter pylori* in gastric mucosa biopsies compared to H&E and Giemsa stains in Kenyatta National Hospital histopathology laboratory?

3.2 BROAD OBJECTIVE

To detect *Helicobacterpylori* in gastric mucosa biopsies using immunohistochemical methods at KNH histopathology laboratory.

3.3 SPECIFIC OBJECTIVES

1. To review the histomorphology of gastric biopsies submitted to KNH histopathology lab.
2. To compare Giemsa-negative *H.pylori* biopsies with Immunohistochemistry.

3.4 SECONDARY OBJECTIVE

1. To assess adequacy of gastric biopsies submitted to the lab.

4.0 MATERIALS AND METHODS

4.1 STUDY DESIGN

A laboratory-based descriptive study.

Thirty gastric biopsies were collected from the KNH Endoscopy Unit. The remaining samples were retrieved from archived blocks in the KNH Histopathology Laboratory.

4.2 STUDY SETTING

Kenyatta National Hospital Histopathology laboratory in conjunction with the UON immunohistochemistry section of the department of Human Pathology.

4.3 SELECTION CRITERIA

4.3.1 Inclusion criteria

All gastric biopsies reported as *H.pylori* negative on Giemsa stain.

4.3.2 Exclusion criteria

1. Biopsies with severe gastric atrophy.
2. Gastric malignancy.

4.4 SAMPLE SIZE DETERMINATION

Sample size was calculated using finite population formula (27) below:

$$n' = \frac{NZ^2P(1-P)}{d^2(N-1)+Z^2P(1-P)}$$

N - total accessible population = 100 per year

Z - standard normal for 95% CI = 1.96

P - Estimated proportion of *H.pylori* in Giemsa negative biopsies = 63%

d - Precision error = 5%

Sample size (n) = 78

4.5SAMPLING PROCEDURE

A sampling frame was generated from the list of gastric biopsies in the laboratory. Simple random sampling technique using a table of random numbers was used to select the biopsies that were included in the study. Selection of the biopsies was performed randomly until the required sample size of 78 was reached.

4.6 SPECIMEN COLLECTION, TRANSPORTATION AND STORAGE

The gastric biopsy was collected by the gastroenterologist. Preservation in 90% formosaline was done immediately.The sample was kept at room temperature and transported to the histopathology laboratory within 12 hours. Processing was done the following morning by the PI and a technologist.

4.7SPECIMEN PROCESSING

i. Samples from the archive diagnosed as chronic gastritis was selected by the PI.

ii. Random sampling was done.

iii. Retrieval of paraffin blocks.

iv. Staining with H&E and Giemsa for:

- review of histomorphologic pattern
- confirming absence of *H.pylori*

v. Cases that meet the inclusion criteria underwent IHC staining.

vi. Screening done by the Principal Investigator.

vii. Reporting findings done with supervisors independently.

viii. For the samples collected from the Endoscopy Unit, a report was availed within one week directly to the clinician by the Principal investigator for further management.

4.8DATA COLLECTION PROCEDURE

a. Data was collected using a structured data tool-vide infra.

b. Age, gender and endoscopy findings were extracted from laboratory request form.

c. Inflammation, bacterial colonization, morphology and background staining were described at microscopy

d. For the purpose of ascertaining reproducibility of the laboratory analysis results and measuring intra-observer variability, a sample of 30 biopsies from the study sample were reviewed by an experienced pathologist in order to determine the inter-observer agreement and as such assess the reproducibility of the results.

4.9RESULT INTERPRETATION

4.9.1 Positive result for H. pylori

The *H.pylori*immunostain stained the bacteria brown while the background consisting of the gastric mucosa stains light purple to white based on cellular constituents.

4.9.2 Negative result for H. pylori

The mucosa without any organisms visibly stained brown was considered negative.

4.10 DATAMANAGEMENT AND ANALYSIS

The data collection tools were coded and entered in Microsoft Access 2013 database designed for the study. Data cleaning was performed continuously during data collection and entry. At the end of data collection, cleaned data was exported to SSPS version 21.0 for statistical analysis. Study population was described using age and sex summarized into mean, standard deviation and percentages respectively. Prevalence of *H.pylori*based on immunohistochemistryresults was analyzed and presented as a percentage with 95% confidence interval. Histomorphology of the specimens was presented as percentages.

Percentage of adequate biopsies was calculated out of all the specimens reviewed and presented as tables, histograms and pie charts.

4.11QUALITY ASSURANCE

- Immunohistochemistry reagents were stored at 2-8°C to maintain stability.
- Pre-diluted reagents were used to avoid dilution errors.

- Pre-charged slides were used.
- Specimen collection and staining for IHC was done according to the protocol.
- Use of controls; both negative and positive.
- Any discordant results were reviewed by a third reviewer - the KNH Pathologist.

4.12 ETHICAL CONSIDERATION

Approval for study protocol was sought from Kenyatta National Hospital/ University of Nairobi –Ethical and Research Committee (KNH/UON-ERC) before the study was conducted.

4.13APPLICATION

The results were forwarded to the gastroenterologist via an ad addendum report for institution of appropriate management for the patient

The findings will be forwarded to various journals forpublication.

5.0 RESULTS

Table 3: Socio-demographic characteristics of patients who had endoscopy

Variable	**Frequency (%)**
Mean age (SD)	48.9 (18.7)
Median (IQR)	50.0 (33.8 - 63.5)
Gender	
Male	37 (47.4)
Female	41 (52.6)

The youngest patient was 7 years old at the time of endoscopy, while the oldest was 92 years. The M:F ratio was 1:1.1.

Table 4: Endoscopy findings of patients who had endoscopy

Variable	**Frequency (%)**
Gastritis	19 (24.4)
Gastric ulcer	10 (12.8)
Duodenal ulcer	6 (7.7)
Gastric polyp	4 (5.1)
Doudenal polyp	1 (1.3)
Normal mucosa	25 (32.1)
Duodenitis	2 (2.6)
Hiatus hernia	3 (3.8)
Not provided	3 (3.8)

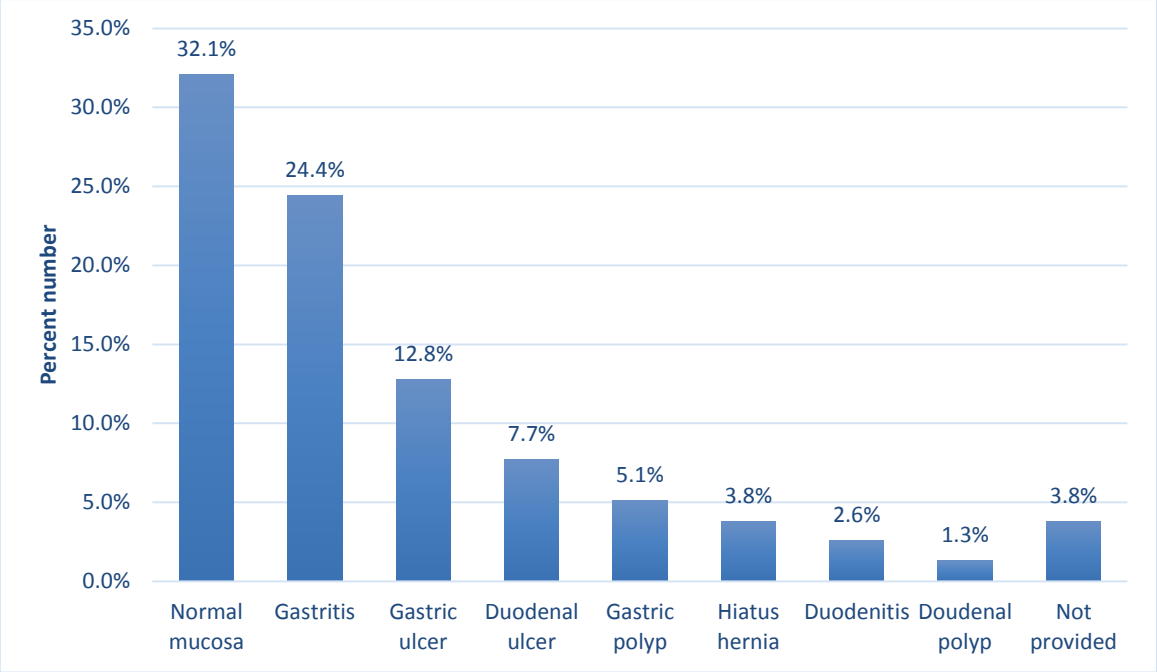


Figure 5: Bar graph 1 - Endoscopy findings of patients who had endoscopy

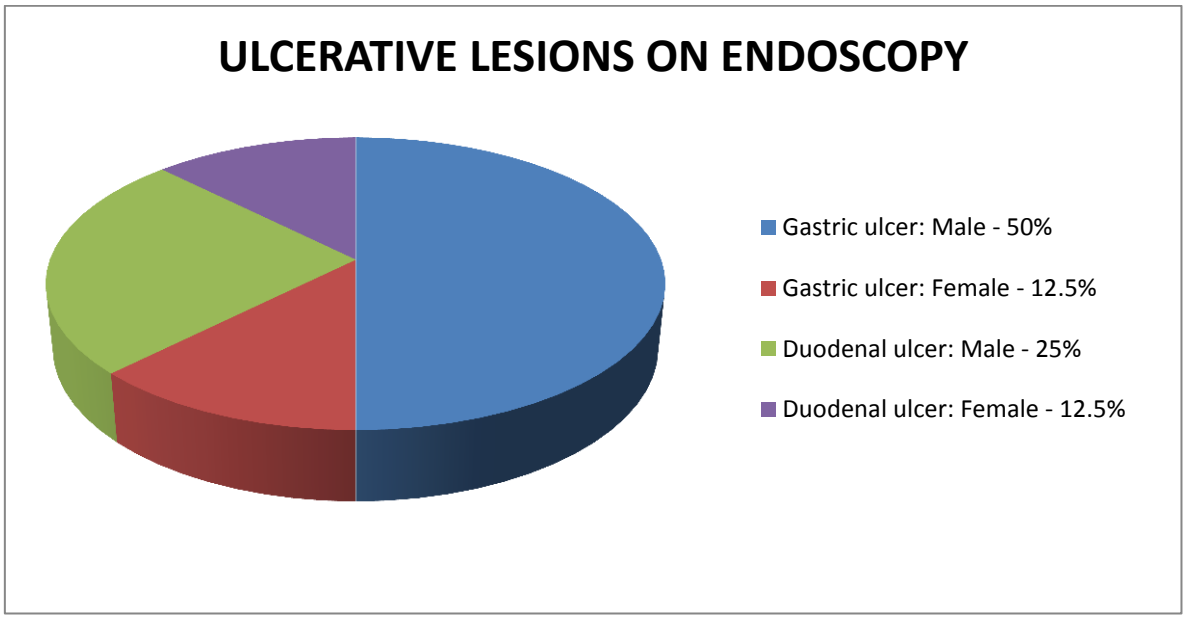


Figure 6: Pie Chart 2 - Male to Female Ratio of Ulcers

Gastric ulcers were present in 8 males and 2 females; with a male to female ratio of 4:1. The ratio of duodenal ulcers was 2:1, with prevalence of 4 males and 2 females.

Table 5: Hematoxylin and Eosin Findings

Finding	Frequency (%)
Type of inflammation	
Chronic inactive	64 (82.1)
Chronic active	14 (17.9)
Severity of inflammation	
Mild	6 (7.7)
Moderate	33 (42.3)
Severe	39 (50.0)
Presence of atrophy	
Yes	10 (12.8)
No	68 (87.2)
Presence of intestinal metaplasia	
Yes	12 (15.4)
No	66 (84.6)
Presence of lymphoid aggregates	
Yes	31 (39.7)
No	47 (60.3)
Presence of mucosal erosions	
Yes	8 (10.3)
No	70 (89.7)
Presence of eosinophilic infiltration	
Yes	13 (16.7)
No	65 (83.3)
Presence of numerous cocci	
Yes	8 (10.3)
No	70 (89.7)

The most common findings were chronic inactive inflammation (82.1%) and severe inflammation (50%). Atrophy, intestinal metaplasia, lymphoid aggregates, mucosal erosions, eosinophilic infiltration and numerous cocci were absent in 87.2%, 84.6%, 60.3%, 89.7%, 83.3% and 89.7% of cases respectively.

Table 6: Immunohistochemistry findings

Variable	Frequency (%)
Helicobacter pylori staining Positive Negative	 20 (25.6) 58 (74.4)
Degree of bacterial colonization 0 +1 +2	 58 (74.4) 17 (21.8) 3 (3.8)
Quality of organism morphology 0 High Medium Poor	 58 (74.4) 1 (1.3) 14 (17.9) 5 (6.4)
Quality of background staining High Medium Poor	 7 (9.0) 65 (83.3) 6 (7.7)

The staining of *H.pylori* was negative in 74.4% of cases. Of the positive cases, 85% showed a low degree (+1) of degree colonization. There was a medium quality of staining of the organisms and background in 17.9% and 83.3% respectively of the total cases. Of the *H.pylori* positive cases, 70% (14 of 20 cases) showed medium quality of organism morphology.

Table 7: Corelation between endoscopy findings and severity of inflammation

Endoscopy finding	Severity of inflammation			P value
	Mild (%)	Moderate (%)	Severe (%)	
Gastritis				
Yes	1 (5.3)	8 (42.1)	10 (52.6)	0.892
No	5 (8.5)	25 (42.1)	29 (49.2)	
Gastric ulcer				
Yes	0	6 (60.0)	4 (40.0)	0.375
No	6 (8.8)	27 (39.7)	35 (51.5)	
Duodenal ulcer				
Yes	1 (16.7)	2 (33.3)	2 (50.0)	0.669
No	5 (6.9)	31 (43.1)	36 (50.0)	
Gastric polyp				
Yes	1 (25.0)	0	3 (75.0)	0.139
No	5 (6.8)	33 (44.6)	36 (48.6)	
Duodenal polyp				
Yes	0	0	1 (100.0)	0.603
No	6 (7.8)	33 (42.9)	38 (49.4)	
Normal mucosa				
Yes	2 (8.0)	12 (48.0)	11 (44.0)	0.759
No	4 (7.5)	21 (39.6)	28 (52.8)	

The findings on endoscopy had no statistical significance with the severity of inflammation (none had *p* value of < 0.05)

Table 8: Corelation between microscopy or endoscopy findings and *H. pylori* staining on -

Variable	Helicobacter pylori staining		OR (95% CI)	P value
	Positive (%)	Negative (%)		
Type of inflammation				
Chronic active	3 (21.4)	11 (78.6)	0.8 (0.2-3.0)	1.000
Chronic	17 (26.6)	47 (73.4)	1.0	
Severity of inflammation				
Mild	0	6 (100.0)	-	
Moderate	9 (27.3)	24 (72.7)	1.0 (0.3-2.7)	0.999
Severe	11 (28.2)	28 (71.8)	1.0	0.930
Presence of atrophy				
Yes	2 (20.0)	8 (80.0)	0.7 (0.1-3.6)	1.000
No	18 (26.5)	50 (73.5)	1.0	
Presence of intestinal metaplasia				
Yes	5 (41.7)	7 (58.3)	2.4 (0.7-8.8)	0.278
No	15 (22.7)	51 (77.3)	1.0	
Presence of lymphoid aggregates				
Yes	12 (38.7)	19 (61.3)	3.1 (1.1-8.8)	0.032
No	8 (17.0)	39 (83.0)	1.0	
Atrophy/severe inflammation/ intestinal metaplasia/ lymphoid aggregates				
Yes	14 (28.6)	35 (71.4)	1.5 (0.5-4.6)	0.441
No	6 (20.7)	23 (79.3)	1.0	
Gastric ulcer				
Yes	3 (30.0)	7 (70.0)	1.3 (0.3-5.5)	0.711
No	17 (25.0)	51 (75.0)	1.0	
Duodenal ulcer				
Yes	2 (33.3)	4 (66.7)	1.5 (0.3-8.9)	0.643
No	18 (25.0)	54 (75.0)	1.0	

The presence of lymphoid aggregates shows positive staining on Immunohistochemistry in 38.7% of the cases; which is statistically significant ($p = 0.032$, OR 3.1)

The severity of inflammation, presence of atrophy, intestinal metaplasia or ulcers has NO statistical significance on immunohistochemical methods ($p > 0.05$)

MICROSCOPY IMAGES

Figure 7: Plate 1 - EXCLUDED CASE No. 1

1(a) shows gastric mucosa exhibiting severe chronic active gastritis with a large lymphoid aggregate in the lamina propria. 1 (b) shows Giemsa staining positive for Helicobacter pylori bacilli (red arrows) in the gastric crypts.

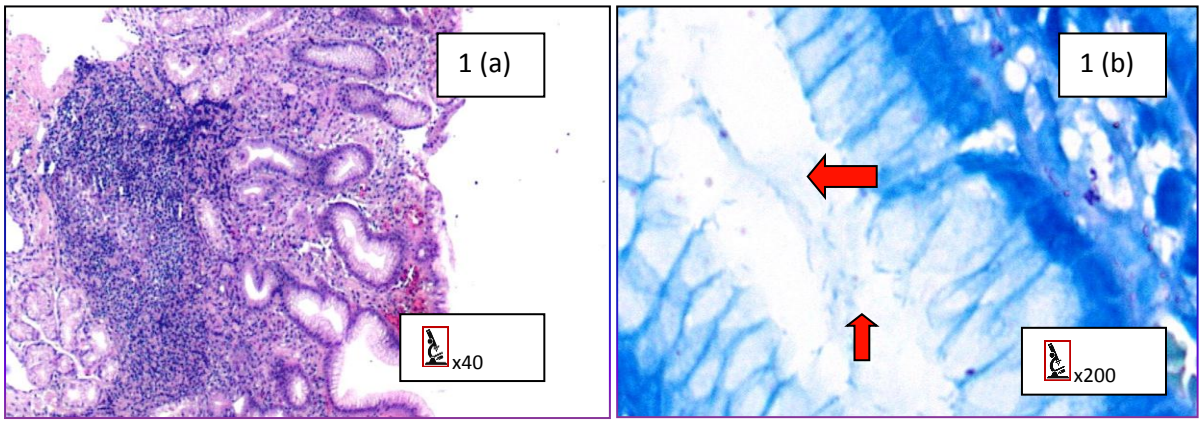
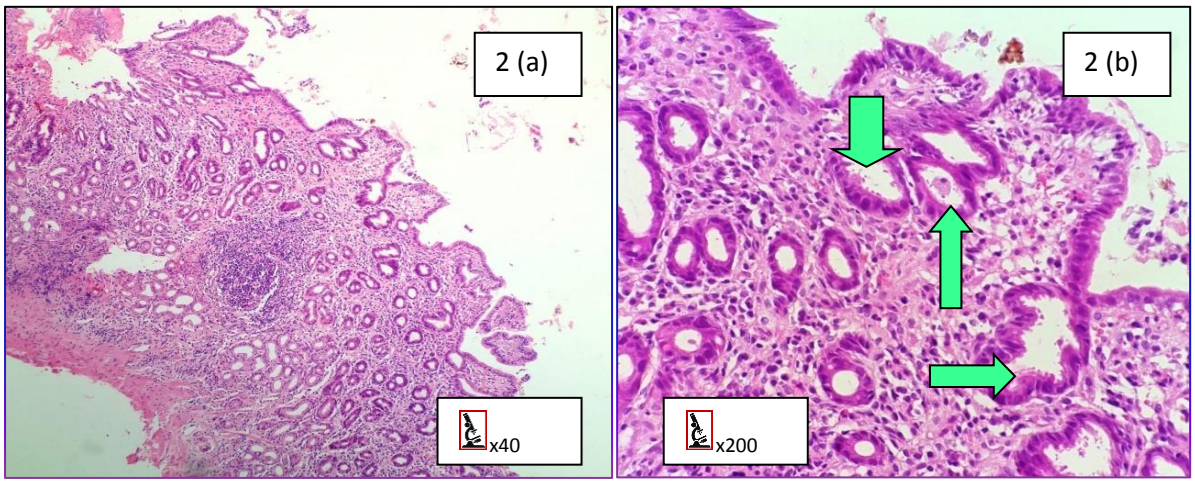
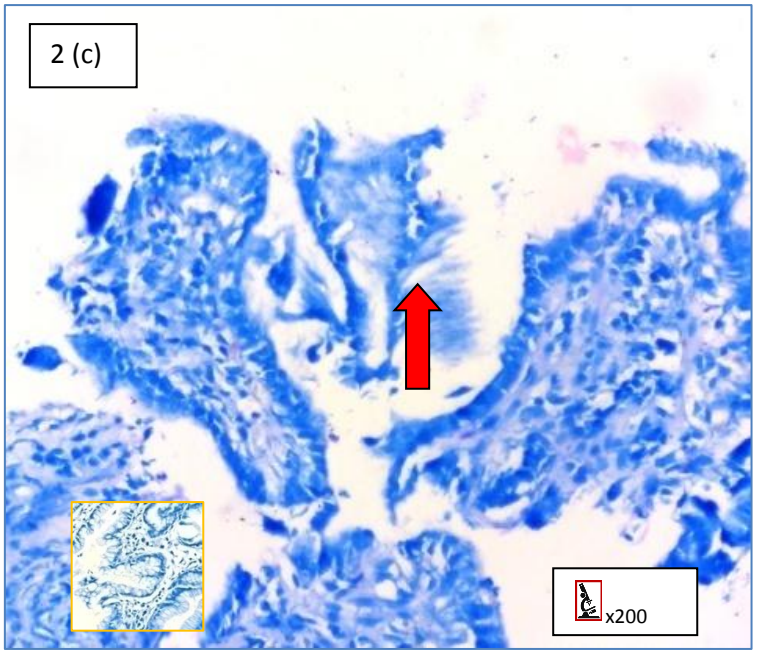


Figure 8: Plate 2 - EXCLUDED CASE No. 8

2(a) shows gastric mucosa with severe chronic gastritis with lymphoid aggregates in the lamina propria. 2 (b) shows gastric glands with Cryptosporidium species cysts (green arrows) and 2 (c) displays *Helicobacter pylori* bacilli (red arrow) in the apical mucus. (Positivecontrolinset)

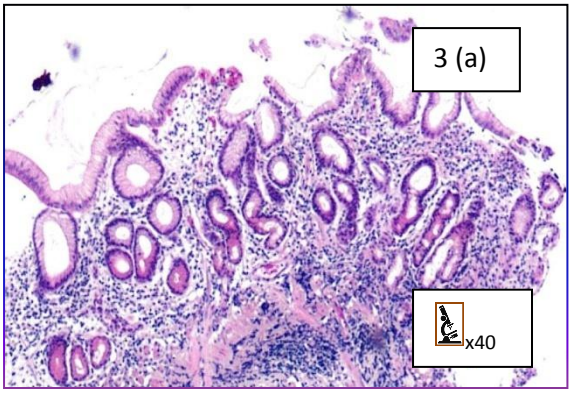


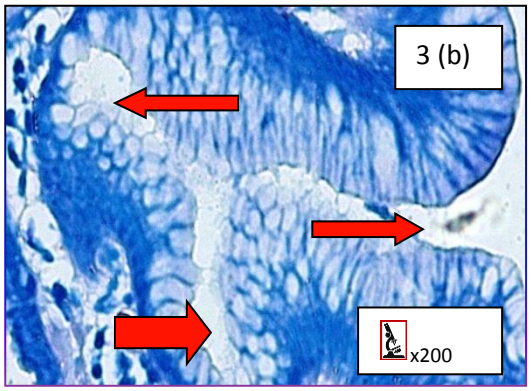


IMMUNOHISTOCHEMISTRY POSITIVE CASES

Figure 9: Plate 3 - CASE 1

3(a) show gastric mucosa with severe chronic active gastritis and *H.pylori* negative on Giemsa stain in 3(b) as evidenced by lack of bacilli visualized in the crypts. In 3(c), immunostaining shows few bacilli on the epithelial surface (green arrow; positive control inset)





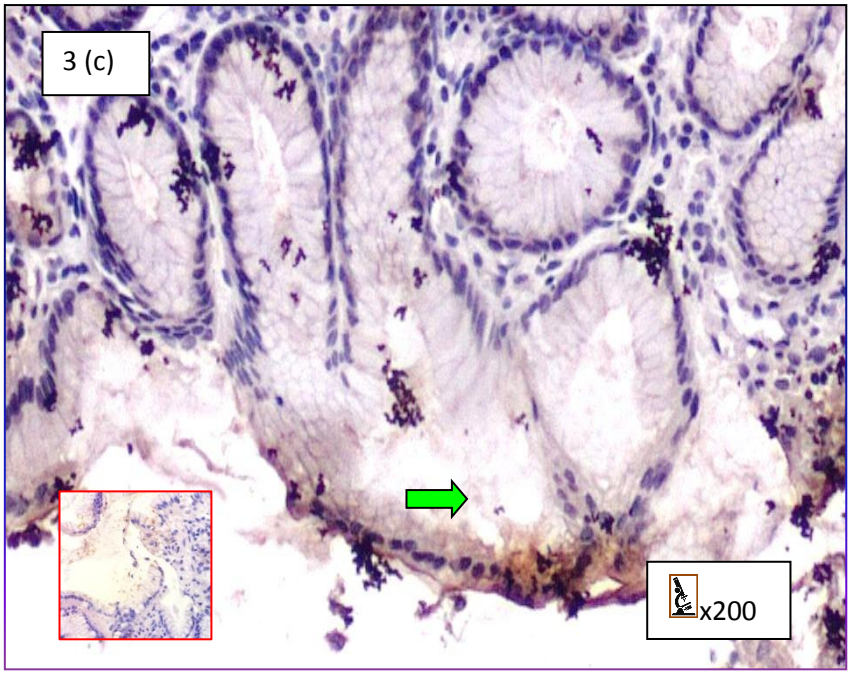
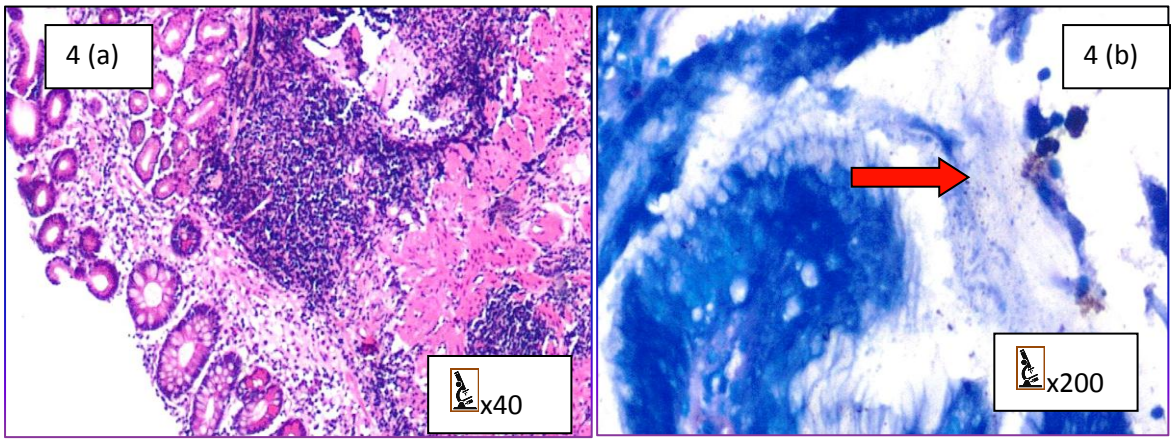
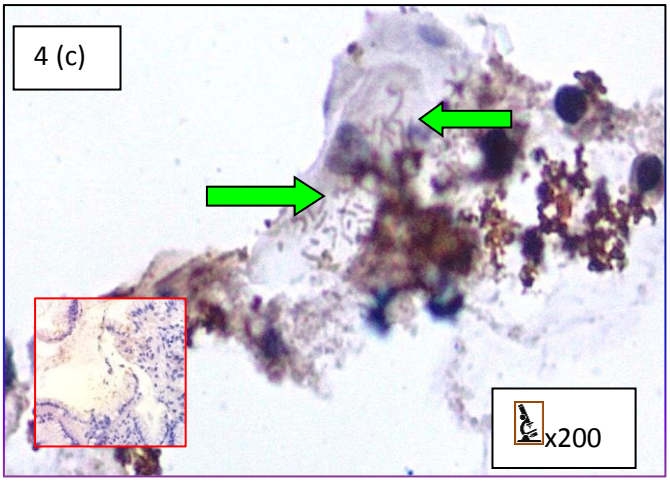


Figure 10: Plate 4 - CASE 66

4(a) shows gastric mucosa attended by severe chronic active inflammation with numerous lymphoid aggregates. Intestinal metaplasia and atrophy are also present. 4(b) shows numerous cocci within the apical mucus (red arrow) staining with Giemsa but not morphologically consistent with *H. pylori*. On use of immunohistochemistry 4(c), bacilli stain readily (green arrows) within the mucus.

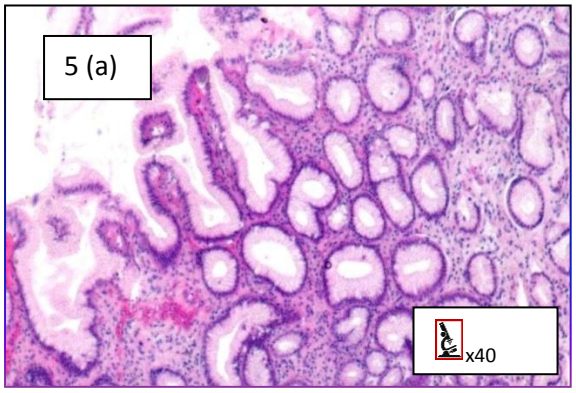


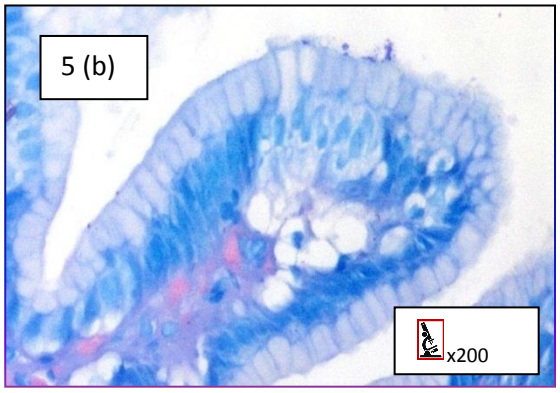


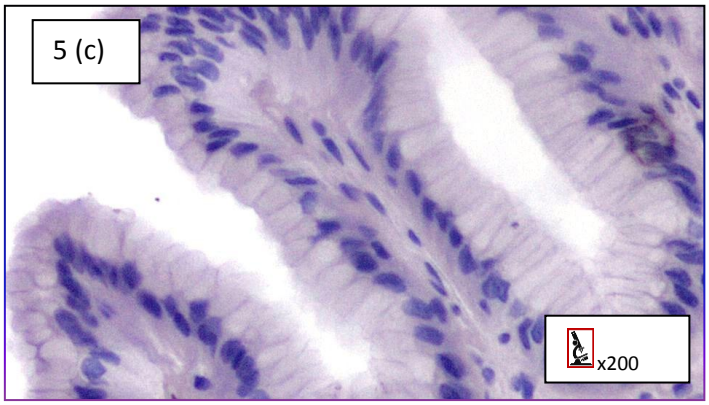
IMMUNOHISTOCHEMISTRY NEGATIVE

Figure 11: Plate 5 - CASE 10

5(a) shows gastric mucosa exhibiting severe chronic inflammation. *H.pylori* is negative on both Giemsa 5(b) and Immunohistochemistry 5(c)







6.0 DISCUSSION

Helicobacter pylori infection is a common cause of gastritis worldwide.Since its discovery by Marshall and Warren, various tests have been developed to diagnose infection. Histopathologic assessment is reliable in detection of the infection and assessing the inflammatory changes that occur in gastric mucosa thereof. Special stains continue to prove vital in visualizing the bacilli and coccoid forms of the bacteria.

The youngest patient was 7 years old and this was the only pediatric case (below 12 years of age). The dearth in the number of pediatric samples submitted to the laboratory should be a cause for concern. This is because it has been shown that there's a higher rate of *H.pylori* infection in the pediatric population in both developing and developed nations(5), (6), (27). This may have affected the overall outcome of positive cases in the immunohistochemistry staining.

The most common endoscopic finding on visual exam was normal mucosa in 25 cases (32.1%). Gastritis was present in 19 cases (24.4%) while duodenitis was present in 2 cases (2.6%). Gastric ulcers and duodenal ulcers were seen in 10 cases (12.8%) and 6 cases (7.7%) respectively and were common in males. Gastric polyps were present in 4 cases (5.1%) and were more common in males while only one duodenal polyp was seen. However, 3 request forms were missing vital data on the gross endoscopic findings. A study by Shrestha et al described common endoscopy findings of gastritis, duodenitis, duodenal ulcer and gastric ulcer with incidences of 63.0%, 11.4%, 12.3% and 11.0% respectively. Normal looking mucosa was not reported in her study as different forms of gastritides were combined into one. Similarly, gastric and duodenal polyps were not reported (28). The two studies show similarity in incidences of gastritis and gastric ulcer while there was a significant difference in the findings of duodenitis and duodenal ulcers; which had a lower rate in this study. This may be due to the large number of study subjects in the Shrestha et al study of 228 cases. Another explanation is that there were 104 patients who were excluded for various reasons, which were not specified, that may have affected their findings.

Hematoxylin and eosin staining easily demonstrated the gastric mucosal changes that occur when the chronic infection is likely present. Of these changes, 64 biopsies (82.1%) demonstrated chronic inflammation without activity. Chronic active inflammation, severe inflammation and eosinophilic infiltration were present in 14 (17.9%), 39 (50%) and 13 (16.7%) cases respectively. These are pointers to recent infection. Eosinophilic infiltration is commonly encountered in children with gastritis. This may occur both in *H. pylori* gastritis and food allergy (30). However, in the one pediatric case in this study, no eosinophilic infiltrate was noted. There likely may be a different etiology inciting the inflammatory response which requires further investigation. One

case demonstrated both Cryptosporidic gastritis and *H. pylori* gastritis (Figure 8). This is likely to occur in the setting of immunosuppression(31). Peptic ulceration was associated with a higher degree of severity of inflammation (+2 and +3). Only one case showed mild inflammation. In patients with non-ulcer dyspepsia (gastritis and normal mucosa on endoscopy), there was a less severe inflammation seen on histopathologic evaluation. Analysis of the endoscopic findings with the severity of inflammation showed no statistical significance.

Giemsa stain is the preferred stain for *H. pylori* diagnosis due to its good sensitivity, excellent specificity, affordability and lack of technical difficulty in preparation. However, as demonstrated by this study, it may fail to accurately diagnose infection. Of the 89 biopsies that were reviewed, 11 cases (12.4%) stained positive on repeat Giemsa stain. These samples had initially been reported as negative for *H.pylori* infection and were subsequently excluded from immunohistochemical staining (Figures 7 and 8) Evidence shows that there is high inter-observer agreement when assessing *H. pylori* density among pathologists (32). Hence, this false negative rate may be explained by poor utilization of positive and negative controls in the KNH histopathology laboratory for Giemsa staining. To further improve detection and quantification of the bacilli, the pathologist is encouraged to examine stained specimen under oil immersion.

Immunohistochemistry is recommended when numbers of the bacilli are too low to detect on H&E and Giemsa stains. It also aids identify cocci in biopsies but aren't diagnostic of *H.pylori* infection on Giemsa stain (24). Of the 78 cases selected for immunostaining, 20 cases (26.5%) were positive on examination. IHC showed a low degree of bacterial colonization (+1) in 85% (17 of 20) of biopsies that were positive. The presence of lymphoid follicles correlated well with positive staining ($p = 0.032$,O.R. 3.1). The severity of inflammation, presence of atrophy and intestinal metaplasia showed no significant correlation with IHC positivity. There are low detection rates where atrophic gastritis and intestinal metaplasia occur, which are unfavorable environments for colonization (29). However, only one case that had numerous cocci seen on Giemsa stain turned positive on IHC.

Based on a study done by Tajalli et al, mild inflammation showed poor detection rates on Giemsa staining (13.0%) whereas there was a higher detection rate of 42.6% on Immunohistochemistry (24). In this study, mild inflammation didn't show IHC positivity in any of the cases. Moderate and severe intensities of inflammation had detection rates of 11.5% and 14.1% respectively. It has been shown that increasing intensity of inflammation shows better detection rates.

On assessment of quality of staining of organisms and the background, a majority of samples had medium quality; despite use of a monoclonal *H.pylori* antibody. Only 1 of 20 cases (2.5%) showing IHC positivity had high quality of organism staining, while only 7 of 78 cases (9.0%) had high quality background staining. Use of Novocastra monoclonal antibody showed high quality staining of organism morphology and background in 75.6% and 95.8% of cases (26). In this study, use of manual immunohistochemical techniques may explain why few cases showed good morphology. However, this is unclear if it affected the overall detection rates. By usingheating method for antigen retrieval rather than trypsin, excessive background staining of epithelium and mucus can be overcome(23).

Failure to note presence of bacilli on special staining techniques where there are characteristic tissue inflammatory patterns has led to the quip; *"seek, yet ye shall not always find."* Therefore, *H. pylori* negative gastritis is considered an entity on its own that requires further investigation (33).Some explanations for this scenario include the use of proton pump inhibitors (which may decrease the numbers of bacteria and shift their populations from the antrum to the corpus), recent use of antibiotics (that may suppress the infection but not reduce the inflammation) and sampling error(29). Other well-known reasons include presence of gastric atrophy and intestinal metaplasia which are adaptive mechanisms to chronic gastritis.In metaplastic areas, *H. pylori* is undetectable by either conventional or special staining techniques in the majority of cases, despite serologic evidence of infection. To reduce the rates of potential false negatives, the following measures are recommended: Adequate sampling from the lesser and greater curvatures, PPIs should be stopped two weeks before endoscopic testing and antibiotics should not be administered four weeks before testing.

A study done by Genta et al explains other factors that result in *H. pylori* negative gastritis. They include other diseases of the gastrointestinal tract such as inflammatory bowel disease and infectious agents. A small proportion of patients who had a negative result, despite their biopsies showing characteristic inflammatory patterns, later showed positive staining on repeat endoscopy after a mean interval of 540days (33). Therefore, further history, proper clinical evaluation and long-term follow up may be recommended for the patients in this study whose results were negative but still have symptoms of chronic gastritis.

In the KNH histopathology laboratory, archived paraffin blocks are properly stored in a secure room. Retrieval was done easily as the blocks are kept in well-labeled carton boxes away from light and heat. Only two blocks that might have been included in the study for retrospective cases were missing. Samples were selected from February 2016 to May 2017. Thirty prospective cases

were collected from August 2016 to May 2017. All the selected archived blocks had sufficient amount of the sample to carry out the three staining techniques. For the prospective samples, processing was carried out as per the laboratory's standard protocols. A total of 89 biopsies that had been diagnosed with chronic gastritis without *H. pylori* infection were selected for review.However, none of the samples had been collected on endoscopy as per the recommendations of the Updated Sydney classification system (19). Therefore, there was a limitation in overall determination of the site of the gastritis requiring use of a modified data entry tool (29).

7.0 STUDY LIMITATION

There was insufficient clinical data found in the request forms that may have affected the outcome of histopathology results. None had indicated whether patients had been on PPIs or antibiotics prior to endoscopic evaluation.

8.0 CONCLUSIONS

1. Hematoxylin and eosin (H&E) stain adequately displays the inflammatory and adaptive changes associated with *H.pylori* infection. Giemsa staining, when performed as per required standards, is the preferred technique to visualize *H.pylori* on gastric biopsies.
2. Immunohistochemistry is a reliable technique and superior to Giemsa stain in detection of *Helicobacter pylori*.It is should be carried out when lymphoid aggregates are present in Giemsa negative biopsies.
3. Gastric biopsies submitted to the laboratory have adequate tissue material for carrying out various staining techniques.

9.0 RECOMMENDATIONS

1. Immunohistochemistry should be introduced in the histopathology laboratory to detect *Helicobacter pylori* infection when Giemsa stain does not detect the bacteria.
2. Gastric biopsy samples should be collected during endoscopy using recommendations from the Sydney system.

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APPENDIX

APPENDIX I: DATA ENTRY SHEET

DATA ENTRY SHEET

- ❑ STUDY NUMBER:_____
- ❑ AGE: _____
  - ☐ Not indicated
- ❑ GENDER:
  - ☐ Male
  - ☐ Female
  - ☐ Not indicated
- ❑ ENDOSCOPY FINDINGS:
  - ☐ Gastritis
  - ☐ Gastric ulcer
  - ☐ Duodenal ulcer
  - ☐ Gastric polyp
  - ☐ Duodenal polyp
  - ☐ Normal mucosa

  Other_____

<u>HEMATOXYLIN AND EOSIN (H&E) FINDINGS</u>

- ❑ TYPE OF INFLAMMATION
  - ☐ Chronic
  - ☐ Chronic active
  - ☐ None

  Other_____
- ❑ Severity of inflammation
  - ☐ Mild
  - ☐ Moderate
  - ☐ Severe
- ❑ PRESENCE OF ATROPHY
  - ☐ Yes
  - ☐ No
- ❑ PRESENCE OF INTESTINAL METAPLASIA
  - ☐ Yes
  - ☐ No
- ❑ PRESENCE OF LYMPHOID AGGREGATES
  - ☐ Yes
  - ☐ No
- ❑ PRESENCE OF MUCOSAL EROSIONS
  - ☐ Yes
  - ☐ No
- ❑ PRESENCE OF EOSINOPHILIC INFILTRATION
  - ☐ Yes
  - ☐ No

- ❑ PRESENCE OF NUMEROUS COCCI
  - ☐ Yes
  - ☐ No

IMMUNOHISTOCHEMISTRY FINDINGS

- ❑ HELICOBACTER PYLORI STAINING
  - ☐ Positive
  - ☐ Negative
- ❑ Degree of bacterial colonization
  - ☐ 0
  - ☐ +1
  - ☐ +2
  - ☐ +3
- ❑ Quality of organism morphology
  - ☐ 0
  - ☐ High
  - ☐ Medium
  - ☐ Poor
- ❑ Quality of background staining
  - ☐ High
  - ☐ Medium
  - ☐ Poor

Signed:

_____

(Principal Investigator)

Date: _____

Signed:

_____

(Supervisor)

Date: _____

APPENDIX II: HAEMATOXYLIN AND EOSIN STAINING

REAGENTS

1. Eosin 1% aqueous solution

Eosin 10g distilled water- 1litres

2. Harris-Haematoxylin solution

Haematoxylin-5g

Ethyl alcohol-50ml

Ammonium alum -100g

Distilled water-1 litre

Mercuric oxide red 2.5g

3. Scotts tap water

Sodium hydrogen carbonate -3.5g

Mgso4 -20g

Distilled water-1 litre

Acid alcohol0.5%Hcl in 70% alcohol

PROCEDURE

1. Dissolve the alum in distilled water heat, stirring frequently.
2. Dissolve the haematoxylin in the alcohol and add to aluminium solution.
3. Bring to the boil while stirring.
4. Mix and allow cooling.
5. Filter into a glass stain bottle and the solution is ready for use.
6. De-wax sections with two changes of xylene.
7. Re-hydrate sections with two changes of absolute alcohol and wash in running tap water.
8. Stain with haematoxylinsolutionforupto 5 minutes.
9. Wash in running tap water.
10. Differentiate in acid alcohol for approximately 5 minutes.
11. Wash in running tap water.
12. Blue in Scotts tap water for few seconds.
14. Wash in running tap water.
15. Stain with eosin for approximately for 5 minutes.
16. Wash in running tap water
17. Dehydrate, clear and mount section.

APPENDIX III: GIEMSA STAINING

REAGENTS

PHOSPHATE BUFFER, pH 6.8:

Sodium diphosphate, 0.3 gm

Sodium monophosphate, 0.7 gm

Distilled water 100.0 ml

GIEMSA STAIN:

- Phosphate buffer 50.0 ml
- Giemsa stain 2.5 ml
- Methanol, acetone free 2.5 ml

Make fresh, filter, discard afteruse.

ACETIC WATER:

- Acetic acid 1.0 ml
- Distilled water 400.0 ml

Stable for 1 year.

PROCEDURE:

1. Deparaffinize, bring to absolute alcohol.

2. Methanol, three changes.

3. Place slide on staining rack, cover with Wright stain, 5 minutes.

4. Do not drain off stain, add an equal amount of distilled water until ametallic sheen appears. Leave for 5 minutes.

5. Place slides directly into the Giemsa solution, for 45 minutes, roomtemperature.

6. Differentiate and dehydrate in the following:

- acetic water 3 dips
- distilled water 2 dips
- 95% alcohol 3 dips
- 100% alcohol 3 dips
- 100% alcohol 3 dips
- xylene 3 changes

7. Place a coverslip

APPENDIX IV: IMMUNOHISTOCHEMISTRY STAINING

DAKO™ LIQUIDMOUSE MONOCLONAL ANTIBODY

STAIN PROPERTIES

Ig Class

IgG1

Total Protein Concentration

1.0–8.0 g/L.

Positive Tissue Control

Recommended positive control tissue is *Helicobacter pylori* infected tissue.If the positive tissue control fails to demonstrate positive staining, results with the test specimens should be considered invalid.

Negative Tissue Control

It should be examined after the positive tissue control to verify the specificity of the labelling of the target antigen by the primary antibody. The recommended tissue is cerebellum.

Non-specific staining, if present, usually has a diffuse appearance. Sporadic staining of connective tissue may also be observed in sections from excessively formalin-fixed tissues. Use intact cells for interpretation of staining results. Necrotic or degenerated cells often stain non-specifically.

Reagents Required but not Supplied

Standard solvents used in immunohistochemistry.

1. 50 mMTris–Buffered Saline (TBS) pH 7.6.
2. Epitope Retrieval Solution (see C. Epitope Retrieval Solutions).
3. Antibody diluent, Novocastra IHC Diluent, RE7133.
4. Visualization system, Novolink5.™ Polymer Detection Systems, RE7280–K (1250 tests), RE7150–K (500 tests), RE7140–K (250 tests) or RE7290–K (50 tests).
5. Mounting medium – use as recommended by manufacturer.

Equipment Required but not Supplied

i. Incubator set to 25°C.
ii. Heating device for epitope retrieval: water bath, steamer, pressure cooker or other temperature controlled laboratory equipment.
iii. General immunohistochemistry laboratory equipment.

PLAGIARISM DECLARATION

Turnitin Originality Report

DETECTION OF HELICOBACTER PYLORI USING IMMUNOHISTOCHEMISTRY AT THE KENYATTA NATIONAL HOSPITAL HISTOPATHOLOGY LABORATORY by Charles Maina Ngari

From Human Pathology (Masters of Medicine)

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Helicobacter pylori: the journey to discovery..

"In 1983, two Australian microbiologists (Warren and Marshall) suggested that gastritis and peptic ulcers were infectious diseases. In the same year the 10th edition of Harrison's Principles of Internal Medicine described peptic ulcers as due to an unfavorable balance between gastric acid-pepsin secretion and gastric and duodenal mucosal resistance.Helicobacter was dismissed because it was so common and its urease considered a secretory product of the stomach itself. Treatment with antacids gave relief, but not cures. Relapsing patients were subjected to surgical treatment (vagatomy, partial gastrectomy) which had their own set of complications. All this was logical and supported by clinical observations and research studies.
It was simply incorrect
This experience has taught us that we can never be smug about what we "know" in medicine"