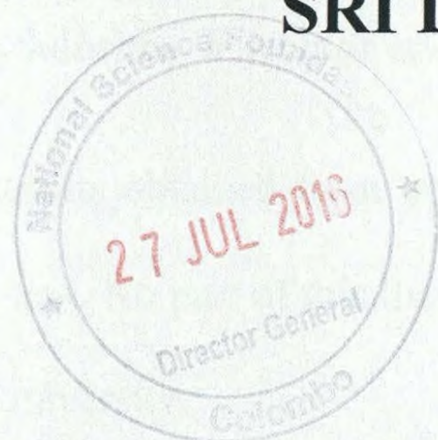
EVALUATION OF COMMONLY USED TESTS FOR DETECTING *Helicobacter pylori* IN A SAMPLE OF SYMPTOMATIC PATIENTS FROM THE CENTRAL PROVINCE, SRI LANKA



By

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Thesis is submitted to the Faculty of Medicine in fulfillment of the requirements for the degree of

Master of Philosophy

Of the

University of Peradeniya

June 2016

DECLARATION

I hereby certify that the work reported in this thesis was carried out by me under the supervision of Dr.Sulochna Wijetunge and Dr.Faseeha Noordeen.

It describes the results obtained from my own work except where due reference has been made in the text. No part of this thesis has been presented for any other degree in this or any other University.

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Acknowledgement

It is with great pleasure that I record my indebtedness to my supervisors Dr Sulochna Wijetunge (Senior Lecturer and Consultant Pathologist, Department of Pathology, Faculty of Medicine University of Peradeniya) and Dr Faseeha Noordeen (Senior Lecturer and Head of Department, Department of Microbiology, Faculty of Medicine University of Peradeniya) for their counsel and guidance for the project, experimental design, laboratory experiments and preparation of this thesis.

I wish to extend my sincere thanks to Dr. Kotakadeniya, Dr Galketiya, Dr. Samarasinghe Dr. Manjula Peiris, Dr. Arenda Dharmapala and the Endoscopy Unint staff nurses of the Teaching Hospital, Peradeniya. I further convey my heartfelt gratitude to Mrs. Siriwardatha and Mrs. Sandiya for helping me to collect the biopsy specimen for the study.

I thank the technical staff of the Department of Pathology, Faculty of Medicine University of Peradeniya, especially to Mrs. Damayantha, Mrs. Nadika, Mrs. Shama and Miss Kowshalya, who helped me with the staining procedure in the laboratory. My thanks also goes to Mr. S.B Abeykoon and Mr.Iriyagahamada from the Microbiology Department.

I would also thank my family and my husband who had been very supportive and helpful throughout for the past four years. I am thankful to National Science Foundation of Sri Lanka for the financial assistance given for the study as well the stipend.

Abstract

EVALUATION OF COMMONLY USED TESTS FOR DETECTING *Helicobacter pylori* IN A SAMPLE OF SYMPTOMATIC PATIENTS FROM THE CENTRAL PROVINCE, SRI LANKA.

Introduction: *H. pylori* gastritis (type II gastritis) is known to have a strong aetiological relationship with benign chronic gastric and duodenal ulcers, gastric atrophy, carcinoma and lymphoma. There are many invasive and non-invasive test methods employed to detect the presence of *H. pylori* in gastric biopsies, i.e., histology, immunohistochemistry, rapid urease tests, culture, urea breath test, stool antigen test, polymerase chain reaction (PCR) and serology. In Sri Lanka, variable prevalence rates of *H. pylori* infection have been reported in different studies ranging from 2.9 to 70.1% using any of the above test methods. Demonstration of *H. pylori* using the histology is the most commonly used method to diagnose *H. pylori* infection in Sri Lanka.

Objectives: The objective of this MPhil project was to evaluate the diagnostic efficacy of commonly used *H. pylori* detection methods in symptomatic patients in Sri Lanka and assess the feasibility of these tests to be adopted in Sri Lankan laboratories.

Methodology: This is a cross sectional study conducted including 205 patients who had dyspeptic symptoms and endoscopically visible mucosal erythema with or without erosions/ulcers at Teaching Hospital, Peradeniya from 2012 to 2013. All patients underwent endoscopic gastric biopsy according to a protocol which included four biopsies from the antrum and one each from incisura angularis and the body of the stomach. The modified one minute rapid urease test was performed on an antral biopsy. The other biopsies were processed for routine histology, toluidine blue staining and immunohistochemistry. On histological examination chronic inflammation, acute inflammation, atrophy, intestinal metaplasia, dysplasia and the presence of *H. pylori* were assessed. PCR was performed on an antral biopsy using primers for the detection of *H. pylori* DNA 16SrRNA. Anti-*H.pylori* antibodies were tested on venous blood using ELISA for anti-IgG antibody (MP Biomedicals, USA) and immunochromatography assay (SD BIOLINE *H. pylori* test, Taiwan). Prevalence of *H. pylori* with each of the detection method was assessed using the test indices such as sensitivity, specificity and positive and negative predictive values.

Results: The sensitivity and specificity of each detection methods were as follows: histology= 54% and 100%, respectively; immunohistochemistry (IHC)= 27% and 100%%, respectively; modified one minute rapid urease test (RUT)=100% and 98.4%, respectively; immunochromatography (ICT)=100% and 96% PCR= 100% and 98.4%, respectively. The overall *H. pylori* prevalence using all detection methods were consistently low, indicating a low prevalence of *H. pylori* in the study population (2.9% using histology; 1.4% using IHC; 5.3% using PCR; 3.4% using ELISA; 6.8% using ICT and 6.8% using RUT).

Conclusion: Overall prevalence of *H. pylori* infection in the study sample was low. Moreover, the low prevalence of serum anti-*H pylori* IgG levels suggested low exposure rate to *H. pylori* in the study sample. Modified one minute RUT has shown to be an inexpensive test with good diagnostic efficacy. Since PCR is a highly sensitive method and using PCR as a gold standard comparator to evaluate other methods could underestimate the method under evaluation. Histology appeared to identify the cases with clinically significant gastric disease. ICT shown to have good diagnostic efficacy and it is a low cost serological test when compared to ELISA.

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LIST OF ABBREVIATION

AP	Activator protein
CLO	Campylobacter like organism
DPX	Di-N-Butyl phthalate in xylene
DAB	3, 3'-Di amino-benzedrine
DNA	Deoxyribo nucleic acid
DNTP	Deoxy nucleoside triphosphate
ELISA	Enzyme linked immunosorbent assay
EDTA	Ethylene Diamine tetra acetic acid
HpSAT	Stool antigen test
ICT	Immunochromatography test
IL	Interleukin
IHC	Immunohistochemistry
MHC	Major histo compatibility complex
NPV	Negative predictive value
PCR	Polymerase chain reaction
PPI	Proton pump inhibitor
PBS	Phosphate buffered saline
PPV	Positive predictive value
RNA	Ribo nucleic acid
RPM	Revolutions per minute
RUT	Rapid urease test
TBE	Tri borate EDTA
TNF	Tumor necrosis factor
TMB	3, 3', 5, 5-Tetra methyl benzedrine
UGIE	Upper gastro intestinal endoscopy
UBT	Urea breath test

Chapter 1 - General introduction and review of literature

1.1 General introduction

Helicobacter pylori infection is regarded as one of the most common bacterial infection in humans and is associated with considerably high morbidity. Long term *H. pylori* gastritis (type II gastritis) is known to have a strong aetiological relationship with benign chronic gastric and duodenal ulcers, gastric atrophy, gastric carcinomas and gastric lymphoma (Warren 1983; Marshal 1985; Graham1989; Dixon 1994). The association of *H. pylori* infection with gastric ulcers is said to be 60% - 70% and with duodenal ulcers almost 100% by serology (Day 2003; Chandrasoma 1999). However, patients who harbour *H. pylori* could also be asymptomatic (Dooley 1990).

Barry Marshall and Robin Warren described the successful isolation of a spiral bacterial species, as *Helicobacter pylori* (Warren 1983) from the human stomach. *Helicobacter pylori* is a common pathogen worldwide. The infections are more common in the low socioeconomic conditions and there are differences in the prevalence of the *H. pylori* gastritis in the different geographical regions (Day 2003; Frenck 2003; Sar 2007). Data from developed countries show a relatively low prevalence of *H. pylori* infection whereas data from developing countries show a high prevalence. The transmission routes of *H. pylori* are still not clearly known. *H. pylori* has a narrow host range and is found almost exclusively in humans and some non-human primates (Brown 2001). Infection with *H. pylori* can be diagnosed using a variety of detection methods and can often be successfully treated with antibiotics.

Different invasive and non-invasive test methods are used to detect *H. pylori* infection and none have been proven to be hundred percent sensitive or specific. Invasive diagnostic tests are rapid urease test, histology, culture and polymerase chain reaction (PCR) and the non-invasive tests include urea breath test, serology for anti *H. pylori* IgG and *H. pylori* stool antigen detection (Calvet 2010). Of the available testing methods, the best method recommended for the detection of gastric *H. pylori* infection is urea breath test and for serological testing is ELISA (Chey 2007; Malfertheiner 2012). In Sri Lanka, varying prevalence rates of *H. pylori* infection have been reported in different studies using different *H. pylori* testing methods, ranging from 2.9% to 70.1%. Histology is the most commonly used test method to diagnose *H. pylori* infection in Sri Lanka. However, the diagnostic efficacy and cost effectiveness of commonly used *H. pylori* testing methods for Sri Lankan laboratories has not been evaluated before. Thus the objectives of the current study focus on determining the efficacy and cost effectiveness of commonly used *H. pylori* testing methods for Sri Lankan laboratories.

1.2 Objectives

1.2.1. General objective

The objective of this MPhil study was to evaluate the diagnostic efficacy of commonly used *H pylori* testing methods in a cohort of symptomatic patients in Sri Lanka and assess their feasibility to be adopted in Sri Lankan laboratories.

1.2.2. Specific Objectives

The specific objectives of this MPhil study were to

1. Assess the diagnostic efficacy of histology against IHC and PCR.
2. Identify the problems associated with histology as a *H. pylori* detection method including inter-observer variations and their influence.
3. Assess the diagnostic efficacy of IHC against PCR and its cost effectiveness for routine diagnostic use.
4. Assess the diagnostic efficacy of modified one minute RUT against histology, IHC and PCR and assess its cost effectiveness for routine diagnostic use in resource limited settings.
5. Assess the diagnostic efficacy of PCR against histology and IHC and its cost effectiveness for routine diagnostic use.
6. Assess the diagnostic efficacy of an ICT (rapid test) for serological diagnosis of *H. pylori* infection against ELISA and assess its suitability to be adopted as a low cost alternative diagnostic method.
7. Determine the prevalence of *H. pylori* infection among symptomatic Sri Lankan patients using all the above test methods.

1.3 Review of literature

1.3.1 History of *Helicobacter pylori*

In 1875, German scientists found spiral-shaped bacteria in the lining of the human stomach, but they were unable to culture these organisms (Blaser 2005). The Italian research team, Giulio Bizzozero (Bizzozero 1893), described similar spiral-shaped bacteria in the acidic environment of the stomach of dogs in 1893. Walery Jaworski from the Jagiellonian University in Kraków investigated sediments of gastric washings obtained from humans in 1899, he also found rod-like bacteria with a characteristic spiral shape, which he called *Vibrio rugula*. He was the first to suggest a possible role of this organism in the pathogenesis of gastric diseases (Physiol 2008).

Helicobacter pylori was re-discovered in the stomachs of patients with gastritis and stomach ulcers in 1982 by Barry Marshall and Robin Warren of Australia. During this period the conventional thinking was that no bacterium can live in the human stomach due to high luminal acidity. In recognition of their discovery, they received the Nobel Prize in Physiology and Medicine in 2005.

In 1979, Robin Warren observed a spiral shaped bacterium in gastric mucosal biopsies from patients with gastric ulcers and he did further research with Barry Marshall in 1981. After numerous unsuccessful attempts, they finally succeeded in culturing bacteria from gastric mucosal biopsies from those with gastric ulcers in 1982. However, they were ridiculed by the scientific community then. In July 1984, Barry Marshall drank a pure culture of these bacteria and induced biopsy proven acute gastritis.

This experiment allowed him to link epidemic gastritis with hypochlorhydria to these bacteria (Marshall 1995). In their original paper, Warren and Marshall contended that most stomach ulcers and gastritis were caused by these bacteria and not by stress or spicy food as had been assumed in the past (Marshall 1984). In 1987 the bacteria was called as *Campylobacter pylori* by Marshall and Goodwin as a result of the frequency of their presence in the stomach and based on the rules of taxonomy (Marshall 1987) and this bacteria is now called as *Helicobacter pylori*. Although *H. pylori* was discovered recently, there is evidence that this is one of the oldest human infections and dated back as the "out of Africa migration event" about 60,000 years ago. Furthermore, geo-genetical diversity of *H. pylori* correlates with the early human migration patterns (David 2001).

1.3.2 Global overview of *Helicobacter pylori* infection

The prevalence of *H. pylori* infection varies widely by geographical areas, age, race and socio-economic status of populations. As this is a chronic infection, it is not possible to know when the infection becomes clinically significant in causing morbidity (Parsonnet 1995). Seroprevalence studies shed information on the diversity of *H. pylori* in geographically and demographically different populations (Shinchi 1997). Half of the world's population is said to be infected by the *H. pylori*. However, the actual infection rates vary from nation to nation. The developing world has much higher infection rates than the West (Pounder 1995). The prevalence in developing countries may reach 70 percent or more compared with 40 percent or less in developed countries (Table1) (Xavier 2013; Linda Morris 2000; The EUROGAST Study Group 1993).

However, it is difficult to compare the rates from these studies as the prevalence rates vary by age and type of the population studied. Children get infected in developing countries at an early age than the developed countries.

The *H. pylori* prevalence by ethnicity and nationality may reflect differences in social and hygienic factors (Malaty 1999). This variability may also be explained by differences in ethnic or genetic predisposition to infections. Although some studies have reported an excess of *H. pylori* in one gender versus the other (Replogle 1995; Smoak 1994), *H. pylori* infection has no noticeable differences in its existence in both genders. Despite high rates of infection in certain areas of the world, the overall frequency of *H. pylori* infection has been declining (Blaser 2006).

Table 1. Prevalence of *Helicobacter pylori* in selected populations in the world.

Country	**Study period**	**Sample size**	**Test method**	**Prevalence %**	**Reference**
Latin America	2009–2012	1852	UBT	79.4	Porras et al 2012
Morocco	2009	429	Biopsy based tests	75.5	Benajah et al 2009
Mexico (Tepehuanos)	2010–2011	156	Serology	66	Alvarado-Esquivel et al 2011
Japan (Nagoya)	1999	4,361	Serology	30	Kikuchi et al 1999
USA (Texas)	2011	281	Serology	17.1	Patterson et al 2011
Nepal	1998	1,142	Serology	57	Kawasaki et al 1998
Korea	1996	161	Serology	75	Malaty et al 1996
USA (Obese)	2001–2009	611	Histology	23.7	Verma et al 2009
Canada	2009–2011	203	Histology	37.9	Sethi et al 2011

In South East Asian countries such as Japan and Korea, *H. pylori* infection rates and its long term complications such as gastric carcinoma incidences are high. In some Asian countries such as India, Bangladesh and Thailand and many African countries, although *H. pylori* infection rates are high, gastric carcinoma incidences are low and are named as Asian and African enigma (David 2001;Hiroto 2002). Differences in the prevalence of different virulent sub-species of *H. pylori* and genetic variations among ethnic groups have been described as possible explanations for these differences.

1.3.3 *Helicobacter pylori* infection in Sri Lanka

Several studies have been carried out in Sri Lanka to assess *H. pylori* infection to elucidate its role in gastritis using different detection techniques. Fernando (1992) has shown a high prevalence of *H. pylori* gastritis of 60% (24/60) in adult patients with peptic ulcer disease and 8% (2/25) with non-ulcer dyspepsia using CLO test. The first isolation of *H. pylori* from a gastric biopsy in Sri Lanka has been reported by Chandrasiri et al (1998) from an 80 year old man with a gastric ulcer and atrophic gastritis. De Silva (1999) has reported a very low incidence of 2.9% (2/67) of *H. pylori* infection in patients diagnosed with functional dyspepsia using CLO test. Fernando et al (2002) has shown 70.1% *H.pylori* prevalence with PCR on 57 Sinhalese dyspeptic patients and 47.5% cag A strain prevalence. Moreover, in 2003, the same group has shown anti-*H.pylori* IgG sero prevalence of 10.3% (37/359) using ELISA (Fernando 2003). Shaman et al (2009) reported a *H.pylori* infection rate of 6.5% by faecal antigen and 27.7% by salivary anti-*H.pylori* IgG among asymptomatic school children.

Fernando et al (2009) has reported a prevalence of 16.7% of *H. pylori* using serology among healthy betel chewers. A study reported by Waidyarathne et al (2010) has shown a *H. pylori* prevalence of 49.40% (124/251) by histology, using Haematoxylin and eosin and Geimsa staining methods) in patients undergoing UGIE. Moreover, another study on histology carried out by Wijetunge et al (2011) has shown a *H. pylori* prevalence of 8.2% (11/134) based on histology in the symptomatic patients attending Teaching Hospital, Peradeniya.

1.4 *Helicobacter pylori*

1.4.1 Taxonomy

H. pylori taxonomically classified as shown below.

Domain:	Bacteria
Phylum:	Proteobacteria
Class:	Epsilonproteobacteria
Order:	Campylobacterales
Family:	Helicobacteraceae
Genus:	Helicobacter
Species:	*H. pylori*

1.4.2 Bacteriology

H. pylori is a helix-shaped, Gram-negative bacteria about 3 µm long with a diameter of ~0.5 µm. It is a motile bacterium and unique in its ability to colonize the normal human stomach. It is microaerophilic and requires 5% of atmosphere oxygen. It produces oxidase, catalase, urease and hydrogenase which can be used to obtain energy by oxidizing molecular hydrogen (H_2) produced by intestinal bacteria (Olson 2002). *H. pylori* can convert its shape from spiral to a viable but non-culturable coccoid form (Chan 1994).

H. pylori have a bundle of unipolar flagella. They are highly motile (Josenhans 2000). The characteristic sheathed flagella filaments of *H. pylori* are composed of two polymerized flagellins, Fla A and Fla B. Each flagella are about 30 nm in diameter with a filament of 12-15 nm. These flagella are well adapted for penetrating the gastric mucous layer and for swimming rapidly in a viscous environment. Moreover, flagella help the *H. pylori* to colonize the mucous layer of gastric epithelium (Rust 2008).

The large amount of urease enzyme production helps the survival of the *H. pylori* in the acidic environment in the stomach. It neutralizes the acid and breaks down the urea present in the gastric to bicarbonate and ammonia. The ammonia is converted to ammonium by accepting a proton (H+) which neutralizes gastric acid. This creates a cloud of acid neutralizing chemicals around the *H. pylori*, protecting it from the acid in the stomach and allows *H. pylori* to colonize in the mucous layer.Two genomes of *H. pylori* have been completely sequenced. The sizes of the two sequenced genomes are approximately 1.7 Mbp, with a G+C content of 35% to 40%. The *H. pylori* strain 26695 genome includes 1,587 genes, whereas the genome of strain J99 includes only 1,491 genes (Boneca 2003; Tomb 1997). Both genomes contain two copies of the 16S, 23S, and 5S rRNA genes. Many strains carry one or more cryptic plasmids, which do not seem to carry antibiotic resistance genes or virulence genes (Heuermann 1995). *H. pylori* is genetically heterogeneous (Kansau 1996), but the differences within related strains may be small.

The genetic heterogeneity helps *H. pylori* to get adapted to the gastric conditions of the host, as well as to the distinct patterns of the host-mediated immune response to *H. pylori* infection (Achtman 2000; Falush 2003; Suerbaum 2004).

1.4.3 Virulence factors of *H. pylori*

Four different virulence factors of *H. pylori* have been identified. These are Cag A, Vac A, Oi pA and Dup A. The cytotoxin-associated gene A product Cag A is the most extensively studied *H. pylori* virulence factor. The main function of Cag A during the pathogenesis is the cellular response and production of cytokines. There are two types of clinical *H. pylori* isolate: Cag A-producing (cag A positive) strains and Cag A-non-producing (Cag A negative) strains. In the Western countries, it has been reported that individuals infected with Cag A positive strains of *H. pylori* are at a higher risk of peptic ulcer or gastric cancer than those infected with Cag A-negative strains (Doorn 1998, Yamaoka 2002). Cag A is a polymorphic gene. There are different numbers of repeat sequences located in the 3′ region of the Cag A gene of different *H. pylori* strains. (Yamaoka 1998,1999 and 2000). The repeat regions were initially classified into two types. The first repeat and the second repeat. Each repeat region contains Glu-Pro-Ile-Tyr-Ala (EPIYA) motifs, including a tyrosine phosphorylation site. The first repeat region is called as EPIYA-A and EPIYA-B segments and the second repeat region as EPIYA-C or EPIYA-D segments (Hatakeyama 2004).

Vac A is a vacuolating cytotoxin and it is the second most extensively studied *H. pylori* virulence factor. In addition to inducing vacuolation in epithelial cells, Vac A can induce multiple cellular activities, including membrane channel formation,

release of cytochrome c from mitochondria leading to apoptosis of the gastric epithelial cells and binding to cell-membrane receptors followed by initiation of a pro-inflammatory response (Atherton 2006; Cover 2005; Kusters 2006). Vac A can also specifically inhibit T-cell activation and proliferation (Boncristiano 2003; Gebert 2003; Sundrud 2004). Virtually all *H. pylori* strains have a functional Vac A gene. There is variation in the vacuolating activity of different *H. pylori* strains (Cover 1992; Leunk 1991) primarily due to differences in the Vac A gene structure at the signal (s) region (s1 and s2) and the middle (m) region (m1 and m2) (Atherton 1995). *In vitro* experiments demonstrated that s1/m1 strains are the most cytotoxic, followed by s1/m2 strains, whereas s2/m2 strains have no cytotoxic activity and s2/m1 strains are rare (Atherton 1995; Letley 1999). There have been many reports showing individuals infected with s1 or m1 *H. pylori* strains to have an increased risk of peptic ulcer or gastric cancer compared with individuals infected with s2 or m2 strains (Doorn 1998; Atherton 1995; Sugimoto 1999).

Oip A is an outer membrane protein, produced by *H. pylori* (Yamaoka 2000). The functional status of Oip A is regulated by slipped strand mis-pairing that is determined by the number of CT di-nucleotide repeats in the 5′ region of the gene (Yamaoka 2000). Oip A was initially identified as a pro-inflammatory-response-inducing protein. One of the functions of Oip A is to induce inflammation and animal studies have revealed that Oip A plays a role in the bacterial colonization of the gastric mucosa (Yamaoka 2002). In 2005, the first disease-specific Dup A gene, was identified as a virulence factor of *H. pylori* (Lu H 2005).It induced duodenal ulcer and had a suppressive action on gastric cancer was identified, and was named duodenal ulcer promoting gene A (Yamaoka 2002).

Virulence factors CagA, VacA and OipA are thought to be involved in the development of both gastric cancer and duodenal ulcer.

1.4.4 Pathogenesis of *Helicobacter pylori* infection

H. pylori is highly adapted to survive in the acidic environment in the gastric mucosa. *H. pylori* has a unique array of features that permit entry into the mucus, swimming and spatial orientation in the mucus, attachment to epithelial cells, evasion of the immune response and as a result, persistent colonization and transmission occur in the gastric mucosa.

To colonize the stomach *H. pylori* must survive the acidic pH of the lumen and use its flagella to burrow into the mucus to reach the niche, close to the stomachs epithelial cell layer. Urease production and motility are essential for these first step *H. pylori* flagella have adapted to the gastric niche (Josenhans 2001). Urease enzyme secreted by the bacterium hydrolyzes urea into carbon dioxide and ammonia thereby neutralizing the acidity in the bacterial micro environment, permitting *H. pylori* to survive in an acidic milieu (Weeks 2000). The enzyme activity is regulated by a unique pH-gated urea channel, UreI that is open at low pH and shuts down the influx of urea under neutral conditions (Mobley 2001). *H. pylori* can bind tightly to epithelial cells by multiple bacterial-surface components (Gerhard 2001). The best-characterized *H. pylori* adhesin, BabA, is a 78-kD outer-membrane protein that binds to the fucosylated Lewis B blood group antigen (Ilver 1998).

The *H. pylori* genome codes for about 1500 outer-membrane proteins (hop proteins).The genome of *H. pylori* changes continuously during chronic colonization of an individual host by importing small pieces of foreign DNA from other *H. pylori* strains during persistent or transient mixed infections (Suerbaum 1998).In the pathogenesis, production of Cag A, Vac A, Oip A and Dup A is linked and the majority of *H. pylori* strains produce either all of these proteins or none of them. The majority of *H. pylori* strains secreted Vac A, an exo-toxin (Montecucco 2001). This toxin inserts itself into the epithelial-cell membrane and forms a hexameric anion-selective, voltagede pendent channel 24 through which bicarbonate and organic anions can be released (Szabo1999) providing the bacterium with nutrients. Vac A is also targeted to the mitochondrial membrane, where it causes release of cytochrome c and induces apoptosis (Galmiche 2000). After entering the epithelial cell, Cag A is phosphorylated and binds to SHP-2 tyrosinephosphatase (Higashi 2002) leading to a growth factor like cellular response and cytokine production by the host cell.

1.4.5 Host response to *H. pylori* infection

The production of ammonia, proteases, Vac A, Cag A and certain phospholipases by *H. pylori* will harm the mucosa in several ways (Smoot 1997). The inflammatory response against *H. pylori* infection initially consists of the recruitment of neutrophils followed by T and B lymphocytes, plasma cells and macrophages along with epithelial cell damage.

When *H. pylori* invade the gastric mucosa, the host response is triggered primarily by the attachment of bacteria to epithelial cells. The *H. pylori* can bind to class II major-histo compatibility-complex (MHC) molecules on the surface of gastric epithelial cells inducing apoptosis (Fan 2000).

H. pylori urease and porins may contribute to extravasation and chemotaxis of neutrophils. Moreover, gastric epithelium will produce enhanced levels of interleukin-1β, interleukin-2, interleukin-6, interleukin-8 and tumor necrosis factor alpha (TNF-α). Among these, interleukin-8, a potent neutrophil activating chemokine expressed by gastric epithelial cells. *H. pylori* strains carrying the Cag A induce a far stronger interleukin-8 response and it produced activator protein 1 (AP-1), this neutrophil-activating protein, may contribute to phagocyte activation.

H. pylori infection induces a vigorous systemic and mucosal humoral response. This antibody production does not lead to eradication of the infection but may contribute to tissue damage. Some *H. pylori* infected patients have an auto-antibody response directed against the H+/K+–ATPase of gastric parietal cells that correlates with increased atrophy of the corpus. Epithelial cell damage results from reactive oxygen or nitrogen produced by activated neutrophils. Chronic inflammation also increases epithelial cell turnover and apoptosis (Sebastian 2002).

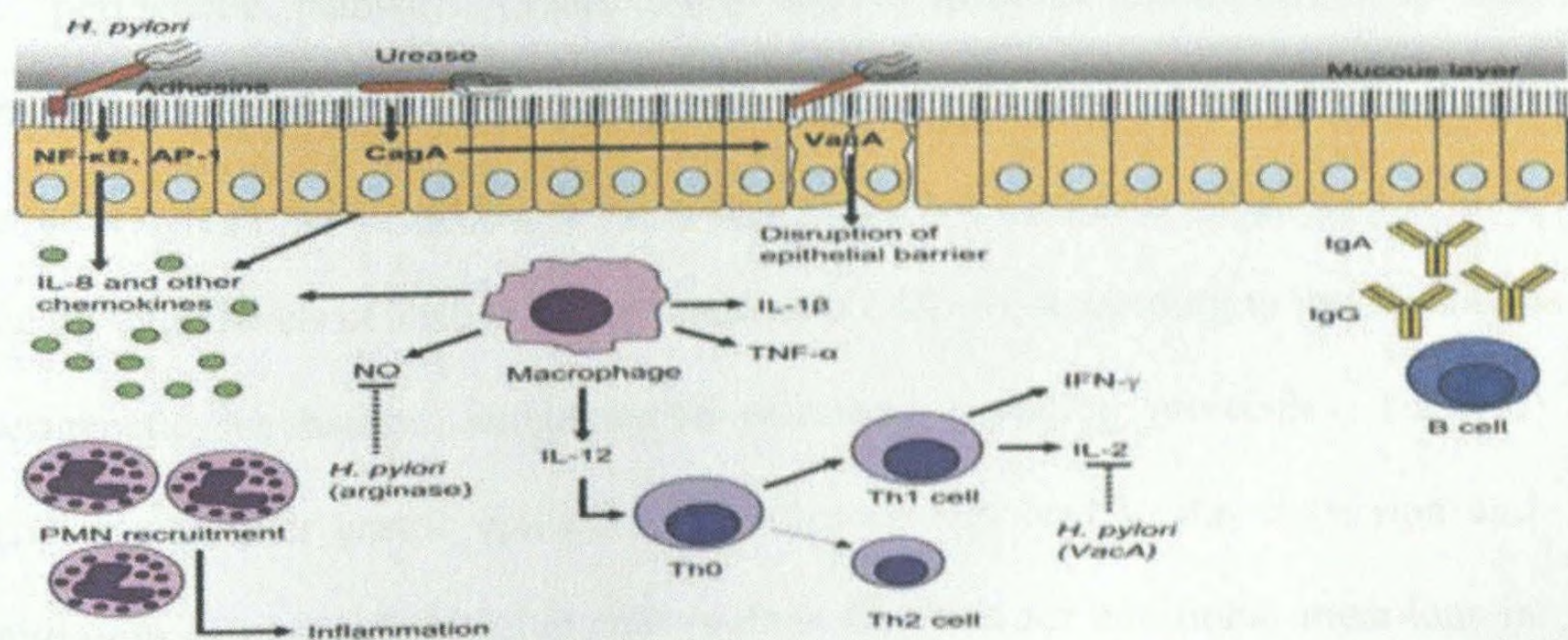


Figure 1. Immune response to *H. pylori* infection in the gastric mucosa (Portal and Perez 2006).

The inflammatory response caused by *H. pylori* colonizing near the pyloric antrum also induces G cells in the antrum to secrete the hormone gastrin, which travels through the blood stream to parietal cells in the fundus (Blasé 2004). Gastrin stimulates the parietal cells to secrete more acid into the stomach lumen and over time it increases the number of parietal cells as well (Schubert 2008). The increased acid load damages the duodenum, which may eventually contribute to the formation of ulcers in the duodenum. When *H. pylori* colonize other areas of the stomach, the inflammatory response can result in atrophy of the stomach lining and eventually ulcers in the stomach. Chronic *H. pylori* infection increases the risk of gastric malignancies such as adenocarcinoma and non Hodgkin lymphoma (Suerbaum 2002).

There are two related mechanisms by which *H. pylori* could promote cancer. One mechanism involves the enhanced production of free radicals near *H. pylori* and an increased rate of host cell mutation. The other proposed mechanism has been called a "perigenetic pathway" (Tsuji 2003) and it involves enhancement of the transformation of host cell phenotype by means of alterations in cell proteins such as adhesion proteins. *H. pylori* have been proposed to induce inflammation and locally high levels of TNF-α and/or interleukin 6 (IL-6). According to the proposed perigenetic 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 tumor suppressor genes, such as genes that code for cell adhesion proteins which causes adenocarcinoma in the stomach (Suganuma 2008).

1.5 Detection of *Helicobacter pylori* infection using different test methods

The diagnosis of *H. pylori* infection is necessary for initiating the eradication treatment. Therefore, several invasive and non-invasive diagnostic tests have been developed and in use. Each test has its advantages and disadvantages and the choice of a test would depend on the clinical assessment of the condition and the availability of the test. Comparison of the accuracy indices of different test methods have given in the table 2.

1.5.1 Invasive methods

Invasive test methods require gastric biopsies through upper gastrointestinal endoscopy and testing using the biopsy tissue include, rapid urease tests, histology, culture and polymerase chain reaction (PCR).

1.5.1.1 Rapid urease tests

The rapid urease tests (RUT) detects active *H. pylori* infection through the urease activity of the bacteria. RUT is a simple, quick and easy to perform test in the endoscopy room. Gastric biopsies are obtained and placed into an agar gel or on a reaction strip containing urea, a buffer and a pH-sensitive indicator, phenol red. In the presence of urease produced by the *H. pylori*, urea is metabolized to ammonia and carbon dioxide leading to a pH increase in the micro environment of the organism. A change in color of the pH sensitive indicator indicates the presence of an active *H. pylori* infection. The results are available within 1–24 hours. There are commercial test kits including CLO test, HP test and pylori-tek test (Basnet 2006). The most widely used test method is the CLO test.

This test consists of a calibrated amount of buffered gel containing urea and a pH indicator with bacteriostatic agents to prevent growth of *H. pylori* and other urease-producing organisms. When the tissue is inserted into the gel, it remains yellow and does not change the color unless urease is present in the biopsy specimen and is observed for 12 to 24 hours. In the original evaluation of the CLO test by Morris and Marshall (2000), a test sensitivity of 95% and specificity of 98% have been reported. Previously also Marshall has reported the same sensitivity and specificity (Marshall 1990). Commercially available CLO test kits are expensive and thus not widely used in developing countries, including Sri Lanka.

One minute RUT described by Arvind et al is an affordable and quick test method, performed using locally prepared reagents (Arvind 1988). Furthermore, one minute RUT has shown to have reasonably high sensitivity and specificity of 91% and 100% (Arvind 1988). Different studies on one minute RUT have reported 96.6% sensitivity 99.2 % specificity compared to CLO test which has a sensitivity of 92% and specificity of 100%. When compared to histology one minute RUT has been shown to have a sensitivity of 99.3 and specificity of 95.3% (Goh 1994).

1.5.1.2 Histology

Haematoxcilin and eosin stain (H and E) combined with a special stain for *H. pylori* such as Geimsa or toluidine blue are the most commonly used histological methods to demonstrate the presence of *H. pylori* infection in gastric biopsies. These are easy to perform routine histological methods available in any histology laboratory. MacMullen, Warthin-Starry, Gimenez, Genta and ancillary stains are some of the special stains used in histology.

However, pathologists generally choose one or more of them based on personal preferences (El-Zimaity 1998; Laine 1997; Rotimi 2000). Many studies have compared H and E stain with other special stains for the identification of *H. pylori* (Pity 2011). Wang et al (2010) concluded that routine H and E staining method were sufficient for identification of *H. pylori*. Toluidine blue is a special stain shown to be much more sensitive than H and E and modified giemsa (Raziye 2013). It is a cheap and easy to use and produces more reliable results than H and E. Toluidine blue is very straightforward, inexpensive and it is more reliable than Hand E for the detection of *H. pylori*. The sensitivity and specificity of toluidine blue stain was 76.74% and 100%, respectively (Raziye 2013). In Sri Lanka, histology is the most widely used tool in detecting *H. pylori* infection. An audit carried out in patients who have undergone UGIE and gastric biopsy for non-malignant gastric lesions during 2009 - 2010 periods shows a low detection rate of 9.7% (3/31) for *H. pylori* in benign gastric ulcers and 13% (6/46) in non-ulcer dyspepsia (Wijetunge 2011).

The reliability of histology in detecting the *H.pylori* depends on several factors, which include the number of biopsies taken, site, size of the gastric biopsy specimens as well as the method of staining and visualizing the *H.pylori* and the expertise of the observer. Moreover, prior treatment with *H. pylori* eradication therapy or antibiotics for other reasons lowers the *H. pylori* density. When the *H. pylori* density is low the inter-observer variability tends to be high. Treatment with proton pump inhibitors is known to induce migration of the organism from the antrum to body, hence, sampling of the antrum may give rise to false negative results.

The significant advantages of histology over other diagnostic test method is the ability to evaluate the pathologic changes associated with *H. pylori* infection such as inflammation, atrophy, intestinal metaplasia and malignancy and this is particularly useful in the case of ulceration when exclusion of malignancy is warranted.

1.5.1.3 Immunohistochemistry

Use of immunohistochemistry (IHC) against specific *H. pylori* antigens is highly sensitive and specific in demonstrating the organisms in tissue sections. IHC is a reliable technique for detection of coccoid *H. pylori* forms, which may not be detectable by other staining methods, but seen easily in IHC stained sections. Moreover, *H. pylori* antigens in lamina propria and beneath the surface epithelia are detectable by IHC, while it can be hardly detectable by routine histochemical stains. On the other hand, IHC is expensive, especially because a positive control needs to be used with every slide. Therefore, IHC is not frequently used in routine diagnostic laboratories. However, in cases with inactive gastritis or low degree of inflammation, IHC stain should be superior to histological staining methods for the detection of *H. pylori* (Ashton 1996).

1.5.1.4 Culture of *H. pylori* in the laboratory

Culture and identification of the isolate is a standard diagnostic technique for most bacterial infections. However, *H. pylori* culture techniques are too demanding with many factors that must be controlled such as micoaerophilic conditions and selective media. Furthermore, the culture takes about two weeks to produce growth. Therefore, culture is not commonly done in the diagnostic laboratories.

Only limited numbers of clinical laboratories perform culture (Cohen 1997). The main advantage of culture in the diagnostic practice is that it allows the characterization of antimicrobial sensitivities. This has become especially important due to emergence of antibiotic resistance in *H. pylori*.

1.5.1.5 Polymerase chain reaction (PCR)

The PCR is a DNA amplification technique that utilizes the rapid production of multiple copies of a target DNA sequence to identify *H. pylori*. PCR has been used for detection and characterization of *H. pylori* from clinical specimens. PCR is regarded as the most sensitive technique for the detection of *H. pylori* in gastric biopsies and it has been evaluated by several investigators and was found to perform well, with sensitivity and specificity usually over 95% as compared to other invasive detection methods (Brooks 2004; Based 1996; Thijs 1996). Because of its high sensitivity, PCR can be particularly useful for the post-treatment evaluation of *H. pylori* status, when the bacterial load is very low. The PCR technique also has made it possible for the detection of DNA in samples that are too small or too degraded to permit other detection methods (Grace 2000).

The goal of PCR is to detect specific DNA sequences rather than the whole viable bacterium and there are no special requirements in transport and storage of the gastric biopsy specimen (Ho 1991; Zwet 1993). Alternatively, specimens embedded in transport media or treated with fixatives do not affect the results of PCR (Greer 1991; Greer 1991). There is no limiting factor with PCR because DNA is chemically stable and can be stored for a longer period without any changes at -80°C. These characteristics make PCR suitable for clinical and environmental sampling and specimens can be transported to the laboratories

without compromising the results of the test (Grace 2000).At present, PCR is still technically demanding and requires expensive infrastructure. Furthermore, its cost effectiveness over more readily available and less expensive tests is being debated. Therefore, PCR is not generally available as a routine clinical diagnostic tool in many developing countries (Grace 2000).

1.5.2 Non-invasive methods

Serology, stool antigen test and urea breath test come under non-invasive test methods for *H .pylori* detection. These non-invasive test methods obviate the need for endoscopy.

1.5.2.1 Serology

H. pylori infection provokes systemic immune response, typically a transient rise in IgM, followed by IgA and IgG throughout the infection. The most commonly used assay is anti- *H. pylori* IgG which typically become present approximately 21 days after infection and usually persist in blood long after eradication (Ho 2000). These antibodies can be detected by different serological methods such as agglutination, immunochromatography, complement fixation and enzyme linked immunosorbent assay (ELISA). These test methods are available as commercial kits which are convenient and simple to perform in most laboratories. Anti- *H. pylori* IgG assay is not likely to be influenced by sampling errors and treatment with colloidal, proton pump inhibitors or antibiotics (Dunn 1997). Due to these reasons, serological tests are used widely (Suerbaum 2002). However, they cannot differentiate an active infection from the past exposure to the *H. pylori* (Vaira 2002) and therefore, cannot be used to assess the response to eradication treatment.

The performance of serological tests depends on the quality of the antigen used (Dunn 1997; Makristathis 2004). Furthermore, *H .pylori* strains differ from each other in different geographical locations thus local validation of the test is important (Suerbaum 2002). Seroprevalence of anti-*H. pylori* IgG of 10.3% has been reported among healthy volunteers in Sri Lanka (Fernando 2003).Several commercial ELISA kits appeared to perform well with sensitivity and specificity values averaging 90-95% and 80-90%, respectively (Youri 1998). The rapid assays e.g. immuno-chromatographic test has an advantage as they can be applied very easily on whole blood obtained by a finger prick and the results are available within 5 to 10 minutes with a simple colour change.

1.5.2.2 Urea breath test

The urea breath test (UBT), like rapid urease tests, identifies active *H. pylori* infection by its urease activity, using the same principle. UBT is the highly sensitive and the most specific non-invasive way of detecting *H. pylori* and is regarded as the best test to detect active *H. pylori* infection (William 2000). In order to detect gastric urease, urea labeled with ^{13}C or ^{14}C is swallowed by the patients. Exhalation of ^{13}C or ^{14}C containing CO_2 indicates the presence of urease activity in patients' gastro-intestinal tract, which indirectly indicates the *H. pylori* infection.

The ^{13}C test is preferred in children and pregnant females (Gisbert 2004). Overall, the performance characteristics of both tests are similar with sensitivity and specificity typically exceeding 95% in most studies (Chey 2000; Gisbert 2004).

The UBT also provides an accurate means of post treatment testing (Leodolter 1999; Chey 2000; Perri 2002). Most tests sutilize a citrate test meal (50–75 mg), which is administered before the labeled urea (Gisbert 2004). Sensitivity of the test is decreased by medications that reduce the density of *H. pylori* and thus urease activity, including bismuth containing compounds, antibiotics, and PPIs. It is currently recommended that bismuth and antibiotics must be withheld for at least 28 days and a PPI for 7–14 days prior to the UBT (Chey 1997). UBT is more costly than the antibody tests or stool antigen test. The expense of the UBT is largely driven by equipmental costs and the cost of labeled urea.

1.5.2.3 Stool antigen test

Stool antigen test (HpSAT) identifies *H. pylori* antigen in the stool by enzyme immunoassay with the use of polyclonal anti-*H.pylori* antibody. Recently a stool test utilizing a monoclonal anti-*H.pylori* antibody has been evaluated (Gisbert 2004; Gisbert 2006). HpSAT is a rapid, non-invasive, easy to perform test that can be used to detect active *H. pylori* infection. It is also a direct antigen test that can differentiate active and latent infection. Furthermore, HpSAT can be used to monitor the effectiveness of therapy and to confirm care after antibiotic use. A study was carried out in 133 patients showed an overall sensitivity and specificity of HpSAT and 13C-urea breath test to be 92.3% and 96.2% versus 88.5% and 99.1%, respectively. In most cases, these studies confirm the excellent performance of the HpSAT before and after therapy (Din 2000).

HpSAT test has received approval from the US food and drug administration for two indications. One was "Diagnosis of *H.pylori* infection in symptomatic adults" and "Monitoring response and post therapy in adults" (Din 2000).

The HpSAT test was first introduced with a polyclonal antibody in 1997, currently many HpSAT tests use monoclonal antibodies (Nakamura 2003). When comparing the polyclonal HpSAT test with the monoclonal HpSAT test; the monoclonal test appears to provide a more reliable means of showing *H.pylori* eradication. The advantages of HpSAT test is easy to use, rapid results, low cost and ease in performing the test in any laboratory. The stool specimen can be obtained with relatively ease from patients who are unlikely to cooperate for endoscopy or breath test. These features makes HpSAT assay, particularly appealing for testing in children. HpSAT test offers a simple, reliable and valid alternative to urea breath test for the diagnosis of active *H. pylori* infection.

Table 2. Comparison of the accuracy indices of different test methods used in the detection of *H.pylori* infection.

Test method	Sensitivity	Specificity	Reference
CLO Test	95%	98%	Morris and Marshall 2000
One minute RUT	91%	100%	Arvind 1988
Histology(toluidineblue stain)	76.74%	100%	Raziye 2013
PCR	<95%	<95%	Brooks 2004; Based 1996
Serology (ELISA)	9%0-95%	80%-90%	Youri 2003
Urea breath test	<95%	<95%	Chey 2000; Gisbert 2004
Stool antigen test	92.3%	96.2%	Din 2000

Chapter 2 - Materials and methods

2.1 Ethical clearance for the study

Ethical clearance for the study was obtained from the Ethical Review Committee, Faculty of Medicine, University of Peradeniya.

2.2 Study population

The study population consist those who presented to the Surgical Clinic at Teaching Hospital, Peradeniya from April 2012 to August 2013 with dyspeptic symptoms and referred for upper gastro-intestinal endoscopy (UGIE) and detected to have "endoscopic inflammation". Endoscopic inflammation was defined as presence of mucosal erythema, erosions or ulcers with UGIE examination.

2.2.1 Inclusion criteria

The inclusion criterion for the study population is presence of dyspeptic symptoms and endoscopic inflammation as defined above.

2.2.2 Exclusion criteria

Exclusion criteria for the study sample is as follows

- Presence of gastric malignancy
- Non consenting patients

2.2.3 Patient recruitment procedure

Patients presenting with dyspeptic symptoms were initially assessed at the Surgical Clinic by a consultant surgeon or a registrar in surgery or a medical officer and those who required UGIE were given appointments. UGIE was performed by a consultant surgeon or a registrar in surgery and assessed for the presence of "endoscopic inflammation". Those who fulfilled the study selection requirements were recruited for the study.

2.3 Experimental design

A cohort of 205 patients was included in the study. All patients recruited underwent a standardized biopsy protocol, blood was obtained for serological studies and clinical and endoscopic data were collected using a standardized questionnaire (Annexure1). Several detection methods were performed to identify *Helicobacter pylori* infections in each patient as given below:

a) Histology based tests
    - Haematoxylin and eosin staining
    - Toluidine blue staining
    - Immunohistochemistry

b) Modified one minute rapid urease test

c) Polymerase chain reaction (PCR)

d) Serology
    - Immunochromatography (SD BIOLINE *H. pylori* test kit).
    - Enzyme-linked immunosorbent assay (ELISA) for detection of *H. pylori* IgG antibodies in the serum.

Informed consent (Annexure 2) was obtained from every patient participated in the study.

2.3.1 Statistical calculation for the sample number

(95% CI) (ME =0.05)

$$n = \frac{P(1-P)Z^2}{ME^2}$$

$$n = \frac{0.117\times(1-0.117)\times1.96^2}{(0.05)^2}$$

n =158.7 ~159.

(95% CI) (ME =0.04)

$$n = \frac{P(1-P)Z^2}{ME^2}$$

$$n = \frac{0.117\times(1-0.117)\times1.96^2}{(0.04)^2}$$

n =248.

The *H.pylori* prevalence rate was 0.117% in the pilot study. Statistically the sample size for the mean of 0.05% was 159 and the mean of 0.04% was 248.Therefor we selected a sample size of 205, which comes in between.

2.4 Clinical data collection

Clinical data were collected using a standardized questionnaire (Annexure 1) from the patient. In the questionnaire (Annexure 1) the following broad details were collected: background information of the patient, clinical symptoms and signs, past medical history including ongoing medications and endoscopic findings.

2.5 Endoscopy, biopsy, sample collection and transport

All patients were advised to fast for 6 hours and were sprayed xylocane (pump spray) to the throat to anesthetize the region. UGIE endoscope (Olympus, Japan) was used to examine the evidence of "endoscopic inflammation" multiple gastric biopsies were obtained using a standardized protocol given below.

Biopsy and sample division protocol for different detection methods

Four biopsies from the mucosal lesion (ulcer, erosion, or erythema) and one each from the antrum, incisura angularis and body were taken for investigation. Biopsies were obtained using a biopsy cutter and a sterilized needle and collected into separate bottles for each location. Gastric biopsies were tested in the following manner.

- One biopsy from the lesion was used to perform one minute RUT at the endoscopy room immediately.
- One biopsy from the lesion was stored at -80 °C for PCR.
- The rest were collected in 10% formaldehyde for haematoxylin and eosin and toluidine blue staining and immunohistochemistry.

2.6 Histology

Histological analysis of the biopsies included two staining methods, namely, haematoxalin and eosin and toluidine blue staining. Immunohistochemistry was performed on formalin fixed paraffin embedded sections.

In the pathology laboratory the biopsy specimen underwent standard processing as listed below.

1. Cut up room procedure
2. Tissue processing
3. Tissue embedding in paraffin wax
4. Sectioning the paraffin wax embedded tissue
5. Staining the tissue section

2.6.1 Cut up room procedure

A dissecting microscope was used for proper orientation of the specimen. This was maintained to obtain a tissue section representing all components of the gastric mucosa (crypts, lamina propria and sculаris mucosa). The size of the biopsy specimen was about 2 mm in diameter and it was wrapped in a piece of tissue paper and placed inside the cassette with a lab reference number for the patient. Biopsy from each gastric location was processed in separate cassettes.

2.6.2 Tissue processing

An automated tissue processor was used for processing of the biopsies. The chemicals used in the tissue processor are 60%, 70%, 80% and 90% of absolute ethanol series, xylene and paraffin wax.

The biopsy tissue in the cassette was placed inside the cassette container in the tissue processor (histokinette) and the total processing time of 14 hours was set. The tissue was made to go through a series of ascending concentration of alcohol to dehydrate the tissue specimen. In the subsequent step the tissue was dipped in 3 xylene containers. Finally the tissue was dipped in melted paraffin wax (57 to 60 ^{o}C) for 45 minutes.

2.6.3 Tissue embedding

The processed tissue, according to the above method was used for the embedding using an embedding system. Initially the processed tissue was kept in a warmer to allow the wax from the processing system to melt. Then the liquid wax was poured into embedding mold which was kept on a warmer. The processed tissue was

Inserted into the liquid wax maintaining a correct orientation. Then the embedding mould was kept on the cooling system for the wax to solidify. The solidified wax was then removed from the mold.

2.6.4 Sectioning the paraffin wax embedded tissue

Sectioning of the paraffin wax embedded tissue required paraffin blocks, microtome blades, rotary microtome, paraffin section mounting bath, mounting needles and slide warmer. The wax embedded tissue was sectioned by a rotary microtome using microtome blades. The tissue sections were cut in 3 µm thick sections. The obtained ribbons of the tissue sections were transferred into a water bath containing water at 58 °C to 60 °C using a mounting needle. The floating tissue sections were observed and the best one without scratches and cracks were selected and transferred onto labeled glass slides. These slides were placed on a rack to drain and transferred to a slide warmer for the paraffin wax to dissolve. Excess wax on the slides was removed by using a cotton wool and the slides were arranged for staining.

2.6.5 Haematoxylin and eosin staining

Composition of haematoxylin and eosin stain contained Harriss haematoxylin and eosin solutions, 1% acid alcohol, 0.2% lithium carbonate (bluing reagent), 90%, 80%, 70%, 60% of absolute ethanol and xylene solution.

Staining procedure

The tissue fixed slides were kept on the slide warmer for a few minutes to de-wax the tissue. The slides were then dipped in a xylene solution for 3 minutes to remove residues.

The above slides were made to go through a series of descending concentration of alcohol (from 90% to 60%) to rehydrate the tissue sections for 3 minutes. The drained slides were dipped into the haematoxylin solution and allowed to stain for 10 minutes and then the slides were rinsed in running tap water. The excess stain was removed by dipping the slides quickly in and out of 1% acid alcohol. Again, it was rinsed in tap water for 2 to 5 minutes to remove the excess acid alcohol.

The water rinsed slides were dipped in 0.2% lithium carbonate to stain the nucleus in blue colour (lithium carbonate intensifies the bluing of the nucleus) and the excess lithium carbonate was removed by rinsing in the running tap water. In the next step, the slides were again dipped and agitated in eosin solution for 1 minute to stain the cytoplasm of the cells in pink colour. Then the slides were dipped in and out of absolute alcohol containers I, II and III to remove the excess eosin. Next, the slides were dipped in xylene solution for 2 to 3 minutes for the clearance of any residual material.

The slides were dried under the warm light for 10 minutes. Finally the slides were mounted using DPX solution with a cover slip and were examined under the microscope. *H. pylori* appeared in pink colour on the surface of the mucus cell layer.

2.6.6 Toluidine blue staining

Toluidine blue stain is a special stain which detects *H .pylori*. Chemicals required for toluidine blue staining include

Solution A containing 1% periodic acid solution; Solution B containing 5g sodium metabisulphate, 1N hydrochloric acid -1mL and distilled water -100mL Solution C containing 1% toluidine blue (aq) -0.5 mL, 3% sodium hydroxide (aq)-2 drops and distilled water-50mL.

Staining procedure

Tissue sections were de-waxed and dipped in the xylene solution and rehydrated by going through a series of descending concentration of alcohol (from 90% to 60%) prior to the staining. One percent (1%) periodic acid solution A was poured over the tissue section on the slide to make the mucus cells to oxidize for 10 minutes. Then the slides were rinsed in running tap water. Solution B was poured over the washed slides for 5 minutes to maintain the oxidized cells. The excess solution removed by rinsing in water for 2 minutes.

Toluidine blue solution C was freshly prepared and the slides were dipped in the solution for 3 minutes. This solution would stain the *H. pylori* bacteria in blue colour. The excess stain was removed by rinsing in water and was blot dried. Then the dried slides were dehydrated in absolute alcohol and cleared in xylene.

Finally the slides were mounted with DPX with a cover slip and were examined under the Olympus light microscope. We did not use alcian yellow stain in our procedure as it was not available.

2.6.7 Immunohistochemistry

Immunochemistry is a sensitive and specific technique to identify antigens in histological tissue sections using antigen specific antibodies. Immunohistochemistry staining was performed by using anti-*H.pylori* antibodies (DAKO B0471). The indirect immuno peroxidase staining technique was used in the test. In this technique the tissue sections were sequentially incubated in two different antibodies. The primary antibody was unlabeled and directed against the antigen under investigation. The envision peroxidase labeled secondary antibody was directed against the primary antigen. The 3,3'-diamino-benzidine (DAB) reagent contained the substrate for secondary antibody and if peroxiadase labeled bound antibodies were present in the section, the peroxidase DAB reaction gave a brown colour. Hence, the presence of *H.pylori* antigen was indicated by staining the organisms in brown colour (Figure 2).

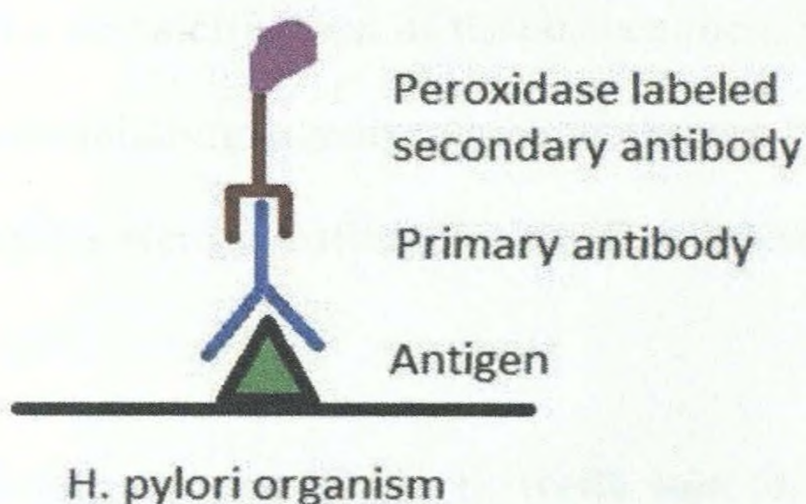


Figure 2. A schematic diagram showing the principle of IHC used for the detection of *H. pylori* antigen in the biopsy tissue sections.

Chemicals required for immunohistochemistry included 2% H_2O_2 solution, absolute ethanol solution, Tris/EDTA buffer, phosphate buffered saline (PBS), 0.1μl goat serum, 0.005μl primary antibody; envision secondary antibody, DAB reagent, carazzine haemotoxylin solution, xylene, poly L lysine and DAKO pen.

Immunohistochemistry staining procedure

The slides were prepared by coating with poly L lysine and de-waxed and rehydrated by alcohol grading prior to staining.

Initially the slides were peroxidase blocked by adding 2% H_2O_2 in absolute methanol for 30 minutes and were rinsed several times in tap water to eliminate the staining of endogenous peroxidase and unblock certain antigenic sites masked during fixation and thus would reduce non-specific background staining. Then the slides were dipped in pre-heated Tris/EDTA buffer and kept it in the microwave oven at 99°C for 20 minutes and the slides were allowed to cool until they reached the room temperature. This would retrieve the antigens. EDTA buffer was used for the de-calcification of tissue specimen. EDTA is also a fixative containing protein-precipitating agents which preserves the immuno-reactivity. After cooling, the slides were transferred to the distilled water. Subsequently, the slides were drained well.

Using the pen (DAKO), wells were drawn surrounding the tissue section on the slides and PBS buffer was applied onto the tissue section to avoid tissue drying. About 0.1μl diluted goat serum was poured over the tissue section and kept for 15 minutes and were blot dried.

The goat serum (DAKO, Denmark) stops the non-specific background staining by blocking sites which show a non-specific affinity for immuno-globulins. Then 0.005μl diluted primary antibody was poured over the tissue sections and was incubated overnight in the refrigerator at 8°C. The next morning the slides were washed with PBS using a magnetic stirrer for 15 minutes and drained. The PBS will wash away all the unbound primary antibodies (Anti-*H.pylori* antibody DAKO B0471,Denmark) before the next immune layer. Envision secondary antibody (Anti-rabbit mouse antibody DAKO, Denmark) was poured over the tissue sections and was incubated for 30 minutes at room temperature. The slides were again washed with PBS buffer solution using a magnetic stirrer for 10 minutes and were drained.

The slides were incubated in the DAB reagent (DAKO, Denmark), which provides the substrate for the peroxidase conjugated in envision secondary antibody for 3 to 6 minutes and was washed well with tap water and drained. Peroxidase–DAB reaction gave a brown colour wherever the enzymes were present. Then the nucleus of the cells was stained by dipping in a Carazzine haemotoxylin staining solution (purple colour) for 4 minutes. After the staining, the slides were rinsed well with water. Finally the slides were air dried and dipped in xylene bath and were mounted with DPX with a cover slip and was examined under the microscope. It was expected that the *H .pylori* antigen would appear in brown colour on the surface of the mucus cell layer. A positive control was used with each batch of samples to ensure the quality of the method at each run.

Review of histology

Each section of the stained slides of haematoxalin and eosin and toluidine blue stains and immunohistochemistry stain were assessed by a histopathologist (principal investigator). Following histological features was assessed and graded from 0 to 3 according to the Sydney grading system (Annexure 3) using their visual analogue scale for (Annexure 4): acute inflammation, chronic inflammation, *H.pylori* density, atrophy and dysplasia.

2.6.8 Inter- observer agreement

Haematoxalin and eosin and toluidine blue stained sections were assessed for the presence or absence of *H. pylori* by three independent pathologists and a senior registrar to assess the degree of inter-observer variation. The instructions given and the format used by the observers are in the annexure (5).

2.7 Modified one minute rapid urease test

The original one minute rapid urease test procedure described by Arvind et al (1988) is as follows: preparation of 10% urea solution by dissolving 10g of urea in 100mL of de-iodinized water having a pH of 6.8. Transfer of 1 mL10% urea solution into a capped eppendorf tube. Immediate transfer of biopsy into the eppendorf tube at the endoscopy room with addition of 2 drops of 1% phenol red to the tube. Recording of a positive result if the colour of the solution in the tube changed from yellow to pink within a minute.

We followed the above procedure with a few modifications to make it more suitable as a low cost user friendly *H. pylori* detection method in the Peripheral Hospitals of Sri Lanka where histopathology services are not available. The modifications adopted in the current study are given below.

1) Instead of freshly prepared 10% urea solution, we prepared a urea stock solution once a week and stored in a domestic type refrigerator. Aliquots of this stock solution were brought to the room temperature before use.

2) Distilled water was used instead of de-ionised water.

3) Solution was observed for a colour change up to 24 hours and interpreted as follows: an obvious colour change from yellow/orange to pink involving the entire test solution within 24 hours was regarded as a positive reaction. Weak colour changes such as, a subtle colour change giving rise to a cloudy appearance or presence of a pinkish smoke like line emanating from the biopsy and no colour change were regarded as negative reactions.

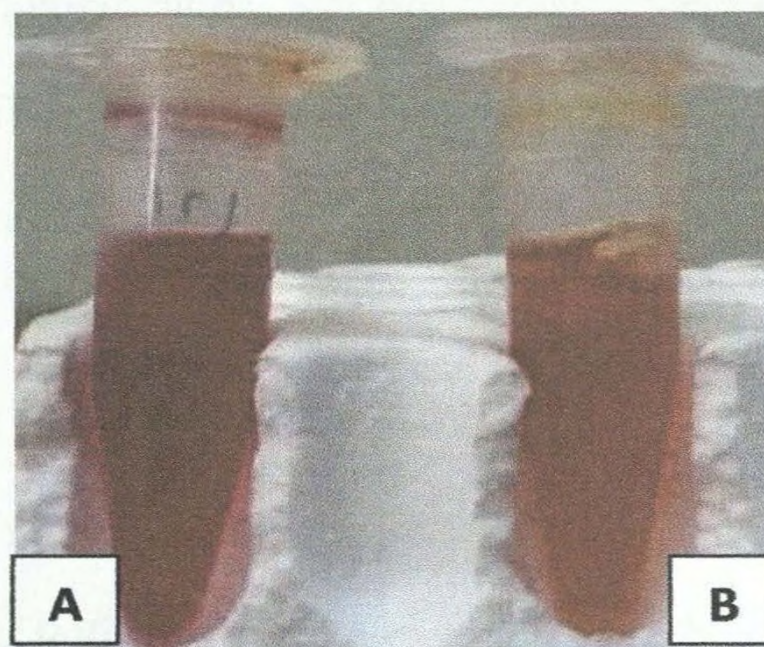


Figure 3. Modified one minute rapid urease test A) positive reaction bright pink B) negative reaction orange.

2.8 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) is a technique, which amplify microgram quantities of DNA copies of the desired DNA segments in a few hours. This method relies on thermal cycling consisting of cycles of repeated heating and cooling of the reaction for melting and enzymatic replication of the DNA. Primers containing complementary sequences to the target region along with a DNA polymeraseare and other key components enable selective and repeated amplification. As PCR progresses, the generated DNA itself will be used as a template for replication, setting a chain reaction in which the DNA template is exponentially amplified. Assays based on PCR technology were employed to detect the presence of *H.pylori* DNA using several target genes including 16rRNA, hpaA, 860 bp DNA fragment and ureC (glmM).

2.8.1 DNA extraction and purification for detecting *H .pylori* DNA by PCR

DNA extraction and purification was done using Pure Link^R Genomic DNA Kits (Invitrogen, USA). Biopsy tissue samples stored at -80°C were brought to the room temperature in 20 minutes. Sterile needles were used to break the biopsy tissue into small pieces.

A volume of 180μl digestion buffer and 20μl proteinase K were added to each biopsy tissue in the eppendorf tube and then the tubes were incubated at 55 °C with occasional vortexing until the lysis was completed. Proteinase K was used for an efficient lysis of the biopsy tissue. The lysate was centrifuged at maximum speed of 13000 RPM for 3 minutes at room temperature and the supernatant was transferred to a micro centrifuge tube.

A 20μl volume of RNase A was added to the lysate and mixed well with brief vortexing and incubated at room temperature for 2 minutes. RNaseA would degrade RNA present in the sample to minimize the RNA contamination. A 200μl volume of genomic lysis/binding buffer was added to the tubes and vortexed for 5 seconds and was ready for binding of DNA.

The lysate was (~640μl) then transferred to the spin column using a pipette and was centrifuged at 10 000 RPM for 1 minute at room temperature. The DNA binds to the spin column and all other molecules remain in the buffer solution. The collection tube was discarded the spin column was placed into a new clean PureLink^{R} collection tube.

A volume of 500μl wash buffer-1 with ethanol was added to the spin column and was centrifuged at room temperature at 10 000 RPM for 1 minute. The collection tube was discarded and the spin column was placed into a new collection tube again. A volume of 500μl wash buffer 2 with ethanol was added to the spin column and was centrifuged for 3 minutes at room temperature at 10 000 RPM and then the collection tube were discarded. The washes would remove all the impurities in the sample.The spin column was placed in a sterile 1.5 mL micro centrifuge tube. A volume of 50μl elution buffer was added to the spin column and was incubated at room temperature for 1 minute and was centrifuged at maximum speed of 13000 RPM for 1 minute at room temperature. After the centrifugation the spin column was discarded and the micro centrifuge tube containing the pure DNA was stored at -20°C.

2.8.2 PCR conditions

The 16SrRNA was selected as the specific gene to detect in the *H .pylori* in the gastric biopsy. The sensitivity and specificity of this gene were 100%, 76%, respectively (Linpisarn 2005). There were forward and reverse primer sets used to target this gene, HP1 (CTG GAG AGA CTA AGC CCT CC) and HP 2 (ATT ACT GAC GCT GAT TGT GC). The 16SrRNA primers yielded the expected product of 110 nucleotide pairs in length.PCR mixture included 5×Green Go Tag Flexi Buffer (pH 8.5), 25mM MgCl2, 10mM of dNTP, 5U/µl Go Tag Flexi DNA polymerase, 0.073 µM of primer HP1 and 0.088 µM of primer HP2. PCR master mix reagents were carried in an ice box into the Pre-PCR room. All the reagents were de-frosted except Tag Flexi DNA polymerase. The PCR master mix was made by adding each reagent in the following order, 6µl of PCR buffer, 2µl of MgCl2, 2µl of dNTP, 2µl of Primer 1, 2µl of Primer 2, 1.5µl of PCR water and finally 0.5µl of Tag polymerase.

The PCR tubes were labeled and 16 µl of PCR master mix was added into each PCR tube. Then 4µl of DNA extract was added to the each PCR tube separately. The loaded PCR tubes vortexes for a few seconds and then placed in the PCR machine. Forty PCR cycles were employed and each cycle consisted of 1 minute denaturation at 95°C and 1 minute at 94°C, annealing at 55°C for 1 minute and an extension at 72°C for 1 minute. After completing 40 PCR cycles, the reaction mixture in the tubes were further extended for 10 minutes at 72°C.

2.8.3 Gel electrophoresis

The PCR products were analyzed by 1.5% agarose gel electrophoresis. A mass of 0.75g agarose gel powder was weighted and was dissolved in 50 mLTris/Borate/EDTA (TBE) buffer and was microwaved for a few seconds. The clear and even agarose gel solution was allowed to cool for a few minutes and then the solution was poured into the gel tray with a comb in place. The gel was allowed to solidify for 20 minutes at room temperature. When the gel was solidified, it was placed into the gel electrophoresis apparatus and the gel apparatus tank was filled with TBE buffer until the gel was covered. A volume of 5μl of 50 bp ladder was loaded into the first lane of the gel and 5μl of PCR products, positive control and negative control was loaded in the subsequent lanes. The gel was run for 20 minutes and then was stained with ethidium bromide for 20 minutes and then visualized under UV light (Alpha innotech, USA). A 110-bp band was considered a positive PCR result for the presence of *H.pylori*. The histology positive cases with *H.pylori* were used as the positive control and water was used as negative control.

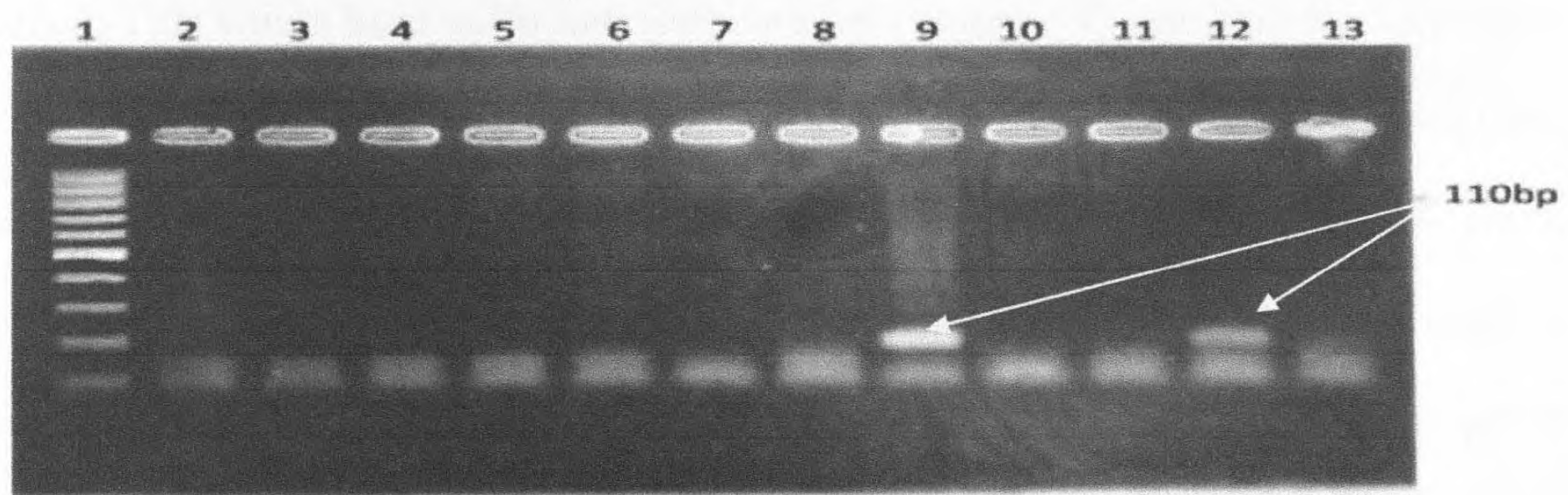


Figure 4. Agarose gel showing the PCR results of 13 cases. Lane 1 is the 50bp ladder. Lane 9 and 12 positive results, indicated by arrows and Lane 13 is negative control.

2.9 Serology

2.9.1 ELISA for detection of anti-*H.pylori* IgG antibodies

Enzyme-linked immunosorbent assays (ELISA) combine the specificity of antibodies with the sensitivity of simple enzyme assays using antibodies or antigens coupled to an easily-assayed enzyme. ELISAs can provide a useful measurement of antigen or antibody concentration. There are two main variations on this method: The ELISA can be used to detect the presence of antigens that are recognized by an antibody or it can be used to test for antibodies that recognize an antigen.

The ELISA test was performed on anti-*Helicobacter pylori* IgG enzyme immunoassay (MP Biomedicals, USA). The microwells were coated with purified *H. pylori* antigens. The diluted serum samples were added to the wells. If the IgG specific antibody was present in the serum it would bind to the antigen coated in the wells. All the unbound materials were washed. Then the enzyme conjugate was

added. This would bind to the antibody-antigen complex. Excess enzyme conjugate was washed off and substrate and chromogen were added. At a specific time enzyme conjugate catalytic reaction was stopped. The intensity of the color generated is proportional to the amount of anti-*Helicobacter pylori* IgG specific antibody in the sample. The results were ready using a microwell reader compared in a parallel manner with calibrator and controls.

ELISA test procedure

The stored serum tubes were taken out from -20°C refrigerator and were kept at room temperature to thaw. After 15 minutes all serum samples in separate tubes were homogenized one by one using a vortex mixture. The serum sample were diluted by according to 1:40 by adding 5 µl of the sample to 200µl of sample diluent and mixed well. The negative control, positive control and calibrator were also diluted to 1:40 by adding 5 µl of the relevant control to 200µl of sample diluent. Diluted human serum containing *H. pylori* specific antigen was used as positive control and diluted human sera was used as the negative control.

A volume of 100µl of diluted samples were dispensed, calibrator and controls were also dispensed into the appropriate wells. Sample diluent of 100µl was dispensed for the blank well in 1A position. Gently tapped the sides of the holder to remove the air bubbles from the liquid and mixed well for 10 seconds and then the ELISA plate was incubated for 30 minutes at room temperature. At the end of the incubation period the liquid was removed from all wells using pipette. The wells were rinsed and flicked 4 times with the diluted wash buffer (1/19) and then once with the distilled water. The micro plate was tapped on the paper towel several times to remove excess liquid on the well. A volume of 100µl of enzyme conjugate was dispensed into each well and was mixed gently for 10 seconds. The plate was then incubated for 30 minutes. At the end of the incubation period the micro wells were rinsed and flicked 4 times with wash buffer and again washed with distilled water. The micro plate was tapped on the paper towel several times to remove excess liquid.

Then a volume of 100µl of TMB reagent was added to each well and mixed gently for 10 seconds. The plate was incubated for 20 minutes at room temperature. At the end of the incubation period, a volume of 100µl of stop solution was added to each well. The plate was mixed gently for 30 seconds and read at an optical density of 450 nm with a microtitter plate reader (Annexure 6).

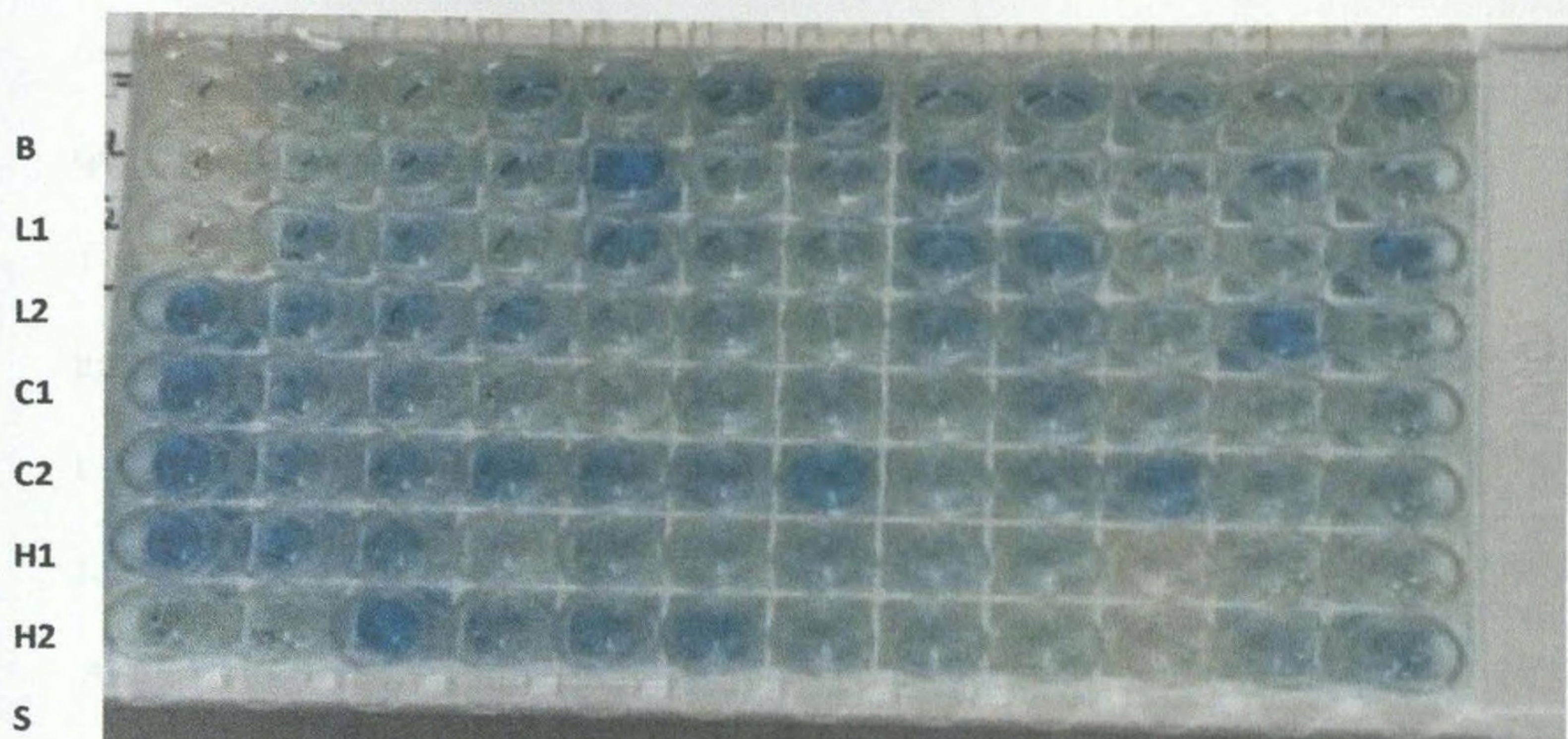


Figure 5. ELISA plate showing the intensity of the color in relation to the concentration of anti-*H. pylori* IgG antibodies present. B - Blank; L1 and L2 - negative controls; C1 and C2 - cut-off calibrators; H1, H2 - positive controls. Rest - Serum samples from the study cohort.

2.9.2 Immunochromatography for anti-*H. pylori* antibodies

Immunochromatography test is carried out using cassettes intended to detect the presence of a target antibody in a sample without the need for specialized and costly equipment or technical expertise. The technique is based on highly specific binding between an antigen and an antibody.

An epitope on the antigen surface is recognized by the antibody's binding site. The type of antibody and its affinity and avidity for the antigen determines assay sensitivity and specificity.

Immunochromatography test method was used for anti-*H.pylori* antibodies detection. The test was carried out using a commercially available rapid quantitative test device (SD Bioline, Taiwan). *H.pylori* test device/multi-device. The test contains a membrane strip, which is pre coated with *H. pylori* capture antigens on test band region. When the serum samples moves along the membrane by diffusion, *H. pylori* antigen colloid and conjugate at the test region (T) and forms a visible line as the antigen-antibody antigen gold particle complex. According to the manufacturer the test has a sensitivity of 95.5% and specificity of 89.6%. The SD *H. pylori* test was performed as a quantitative detection of anti-*H. pylori* IgG, IgM, IgA specific to *Helicobacter pylori* in human serum.

Immunochromatography test procedure

The stored serum tubes were taken out from -20°C refrigerator and were kept at room temperature to thaw. After 15 minutes all the serum tubes were homogenized using a vortex mixture.

The assay cassettes were labeled. A volume of 10 µl serum was pipetted from each serum tube and was added to the each sample well in the assay cassette and three drops of assay diluent was added to the same assay cassette. After 10 minutes assay cassette was observed.

Two purple colour parallel lines (T), (C) appeared on the cassette indicated a positive result. The appearance of one purple line (C) indicated a negative result.

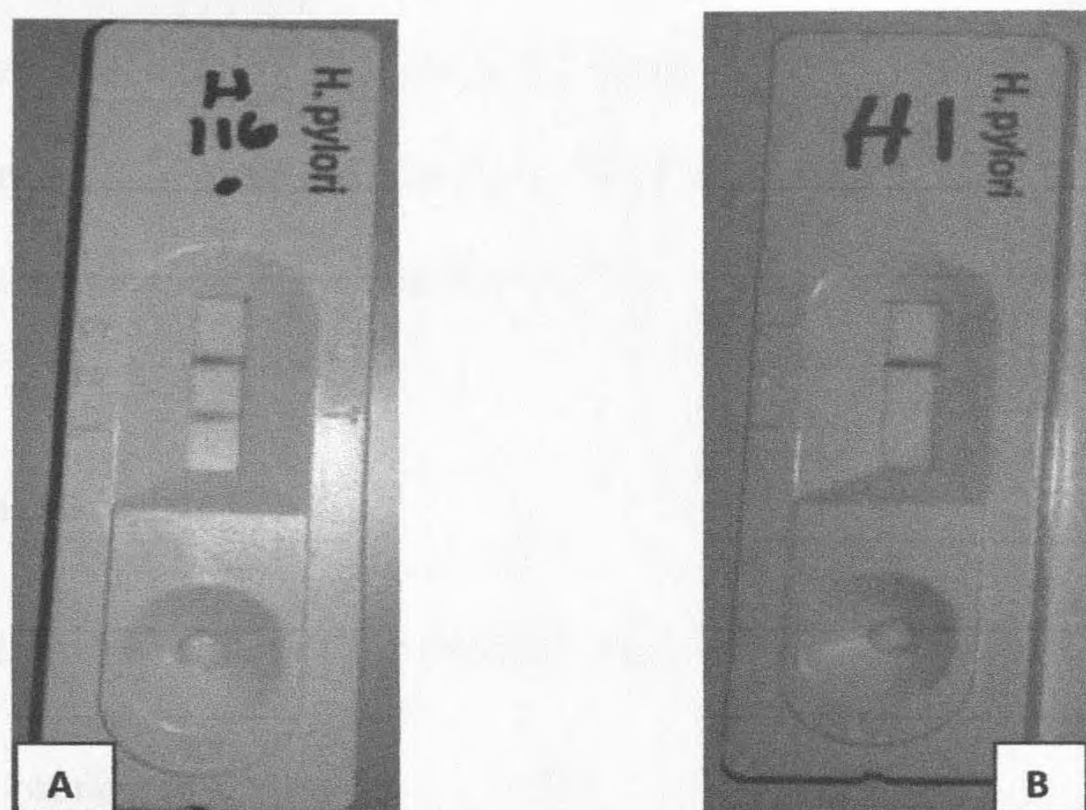


Figure 6. Immunochromatography assay for *H. pylori* antibody detection. A - Positive test showing visible T and C line; B - Negative test showing only C line.

2.10 Analysis of data

Diagnostic efficacy of tests was analyzed in terms of sensitivity, specificity and positive (PPV) and negative predictive values (NPV). Histology review results of the principal investigator were used for analysis of diagnostic efficacy.

The inter-observer variations of the histological results were analyzed using inter-class correlation coefficient. The confidence interval was set at 95% for all statistical methods used. Furthermore, the data were analyzed to investigate the problems associated with histology as a test for detecting *H. pylori* and the measures that can be employed to improve its suitability in the same sample

Chapter 3 – Results

A total of 656 patients underwent upper gastrointestinal endoscopy during the study period and 205 (31.3%) subjects fulfilled the inclusion criteria for the study. The mean age of the patients was 56 ± 14 years (range = 22 - 89 years). There were 116 (56.5%) males and 89 females (43.3%).

3.1 Clinical presentation

Table 3. Clinical profiles of the patient cohort investigated for the study

Post prandial fullness	150
Acid bash	106
Early satiety	137
Regurgitation	140
Epigastric pain or burning	137
Restrosternal pain	112
Haematemesis	36
Melena	79

None had been treated for *H. pylori* eradication (triple therapy) during the preceding one year. Table 3 indicates the different symptoms present in the patients in the study.

3.2 Endoscopic appearances

The endoscopic findings of 205 patients were as follows: 59 ulcers, 58 erosions, 78 inflammations and 11 pan gastritis. The endoscopic appearance of the patients in the study is shown in Figure 7.

A total of 59 patients had ulcers, of them, 24 had antral ulcers, 30 had pyloric ulcers, 4 had body ulcers, and one a cardiac ulcer. Ninety patients had erosions of which 57 were antral erosions and 15 were erosions involving the entire stomach. Five erosions were seen in the body and 7 erosions were seen in the fundal region of the stomach. Moreover, 5 patients had pyloric erosions and only one had cardiac erosion.

A total of 150 patients had antral inflammations, of them, 135 had inflammation only in the antrum and 3 had inflammations in the antrum and pyloric region of the stomach. One had inflammation in the body, 2 had inflammations in the fundal region.

Two patients had only gastric mucosal inflammation, 4 had pyloric inflammation and 3 had inflammation throughout the stomach. Only 11patients had pan gastritis.

Figure 7. Upper gastrointestinal endoscopic views of manifestations of gastric mucosal inflammation (arrows). A - erythema; B - erosions; C - ulcer.

3.3 *Helicobacter pylori* detection

Table 4. Total positive results of the Different test methods used in detection of *H. pylori* infection.

Test methods used	No of positives	% positives
H & E histology	5	2.9%
Toluidine blue histology	6	2.9%
Immunohistochemistry	3	1.4%
Rapid urease test	14	6.8%
PCR	11	5.3%
ELISA	7	3.4%
Immunochromatography	14	6.8%

3.4 Histology results

Out of 205 patients, 5 were positive with haematoxalin and eosin staining based histology alone and 6 were positive for toluidine blue staining based histology. There was only one patient had evidence for intestinal metaplasia and none of the patients had atrophy or dysplasia.

Table 5. Histology based results for *H. pylori* infection in correlation with the other tests.

Histology	**Histology with H & E and toluidine blue staining**								**IHC +ve**	**Modified one minute RUT +ve**	**PCR +ve**	**ELISA +ve**	**ICT +ve**
	Chronic inflammation				**Acute inflammation**								
Sydney grade system	**0**	**1**	**2**	**3**	**0**	**1**	**2**	**3**					
Histology +ve 6	-	1	3	2	3	1	2	-	3	6	3	5	4
Histology -ve 199	140	44	10	-	188	4	2	-	0	8	9	2	10

H & E - haematoxylin and eosin; IHC - immunohistochemistry; RUT - rapid urease test; PCR - polymerase chain reaction; ELISA - enzymelinked immunosorbent assay; ICT - immunochromatography test.

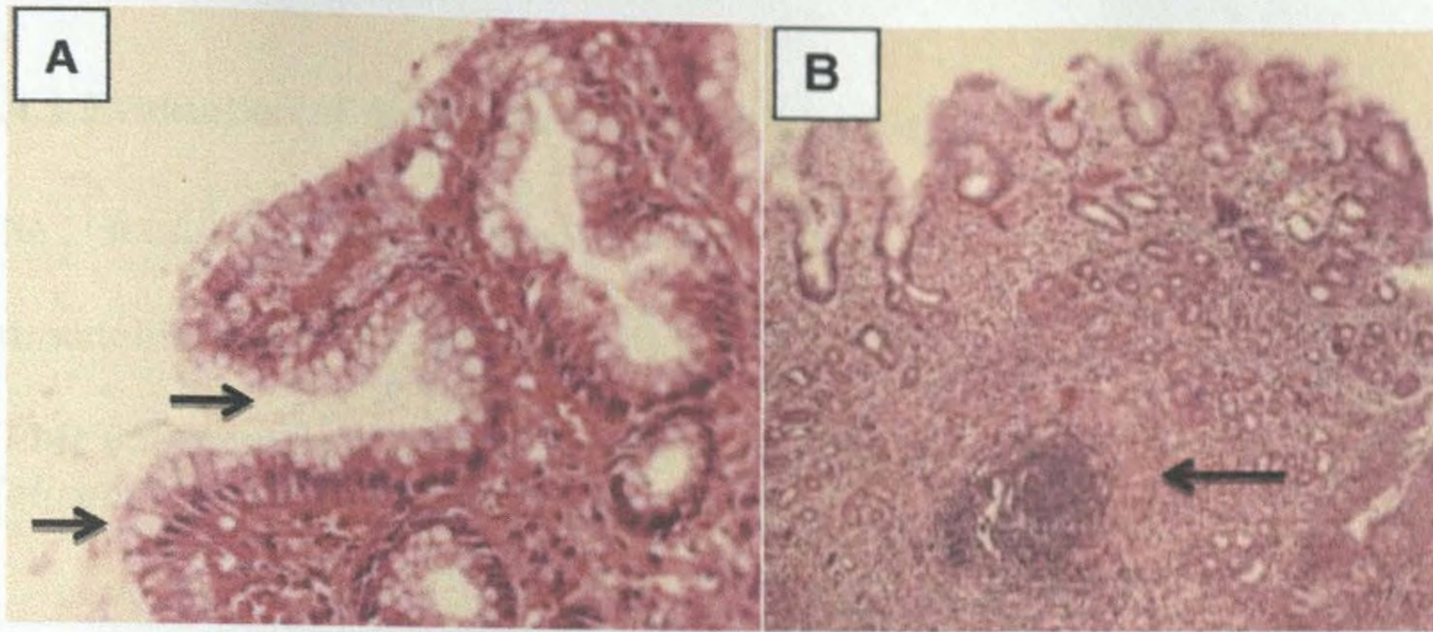


Figure 8. The typical histology of *H. pylori* gastritis. A - H & E stain demonstrating the presence of *H. pylori* in the mucus layer of the stomach. B - Severe chronic inflammation in the lamina propria. Arrow in B indicates a lymphoid follicle.

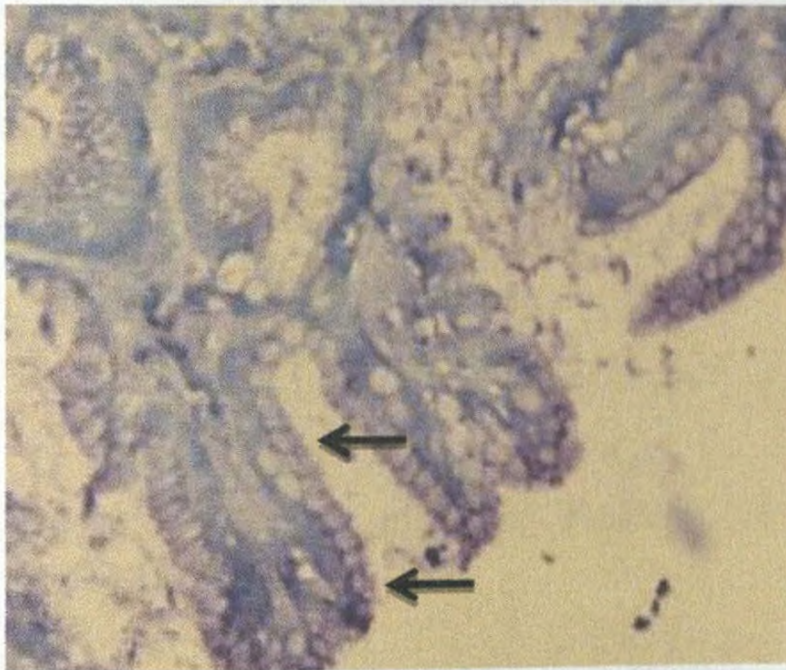


Figure 9. Toluidine blue staining demonstrating the presence of *Helicobacter pylori* in the mucus layer of the stomach. Arrows indicate the presence of *H. pylori*.

3.4.1 Evaluation of diagnostic efficacy of histology

The sensitivity and specificity of histology were evaluated against immunohistochemistry and PCR (Table 8).

Table 6. Comparison of histology with immunohistochemistry for the detection of *Helicobacter pylori* infection.

	IHC +ve	IHC -ve	Total
Histology +ve	3	3	6
Histology -ve	0	199	199
Total	3	202	205

Table 7. Comparison of histology with PCR for the detection of *Helicobacter pylori* infection.

	PCR +ve	PCR -ve	Total
Histology +ve	6	0	6
Histology -ve	5	194	199
Total	11	194	205

Table 8. Accuracy indices of histology for the detection of *Helicobacter pylori* infection

	Sensitivity	Specificity	Positive predictive value	Negative predictive value
Histology against IHC	100%	98.5%	50%	100%
Histology against PCR	54.4%	100%	100%	97.4%

3.4.2 Statistical analysis of inter-observer variation of histology

Table 9. *H. pylori* detection with H & E and toluidine blue by 4 observers.

Observer	H & E	Toluidine blue
A	12 (5.8%)	21 (10.2%)
B	3 (1.4%)	4 (1.9%)
C	22 (10.7%)	38 (18.5%)
D	5 (2.4%)	6 (2.9%)

Table 8 provides *H. pylori* detection rates by the four observers. Intra-class correlation coefficient (ICC) was calculated to assess the degree of inter-observer agreement. The ICC value for H & E stain for single measures was 0.157 (95% CI, 0.069 – 0.23) and average measures 0.428 (95% CI, 0.228 – 0.588). The ICC value for toluidine blue stain for single measures was 0.105 (95% CI, 0.023 – 0.208) and average measures 0.320 (95% CI, 0.085 – 0.513). The inter-observer correlation in detecting *H. pylori* infection with H & E and toluidine blue stain were not satisfactory when considering an ideal agreement of ≥ 0.8.

3.5 Immunohistochemistry results

Out of 205 patients only 3 became positive with immunohistochemistry.

Table 10. Immunohistochemistry results in correlation with the other tests for the detection of *Helicobacter pylori* infection.

IHC	H and E +ve	Toluidine Blue +ve	Modified one minute RUT +ve	PCR +ve	ELISA +ve	ICT +ve
IHC +ve = 3	3	3	3	2	3	3
IHC-ve = 202	-			-	-	-

H & E - haematoxylin and eosin; IHC - immunohistochemistry; RUT - rapid urease test; PCR - polymerase chain reaction; ELISA - enzymelinked immunosorbent assay; ICT - immunochromatography test.

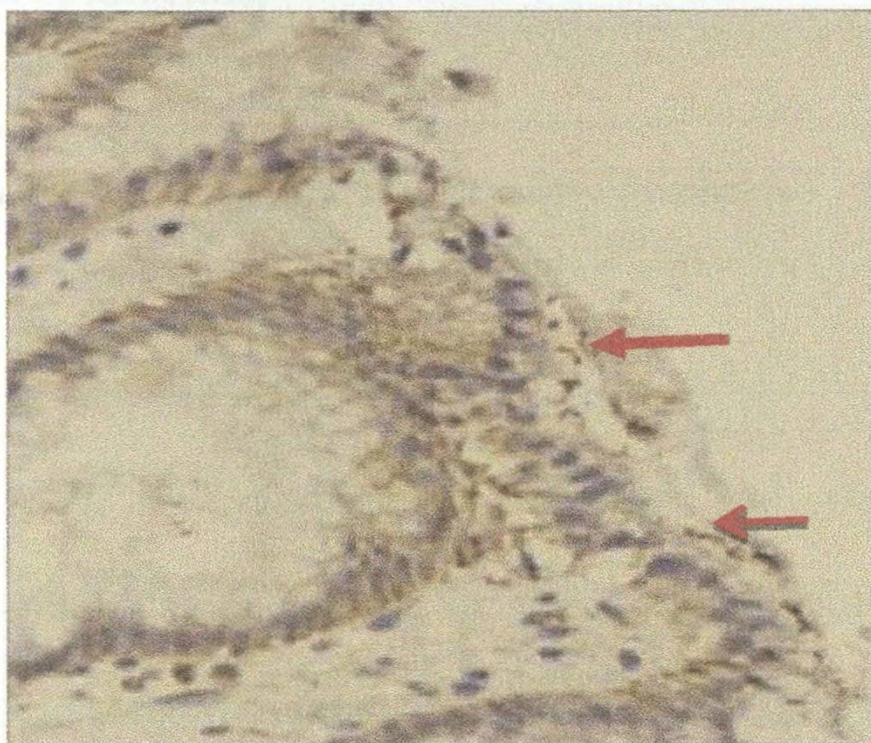


Figure 10. Immunohistochemistry staining demonstrating the presence of *Helicobacter pylori* in the mucus layer of the stomach. Arrows indicate the presence of *H. pylori*

3.5.1 Evaluation of diagnostic efficacy of immunohistochemistry

The sensitivity and specificity of immunohistochemistry were evaluated against PCR (Table 12).

Table 11. Comparison of immunohistochemistry with PCR for the detection of *Helicobacter pylori* infection.

	PCR +ve	**PCR -ve**	**Total**
IHC +ve	3	0	3
IHC-ve	8	194	202
Total	11	194	205

Table 12. Accuracy indices of IHC against PCR for the detection of *Helicobacter pylori* infection.

Sensitivity	**Specificity**	**Positive predictive value**	**Negative predictive value**
27.2%	100%	100%	96%

All *H. pylori* positive patients had positive results with histology and immunohistochemistry in the biopsy from the lesion. Inclusion of additional biopsies from the incisura and body did not increase the detection rate.

3.6 Modified one minute rapid urease test results

Out of 205 patients, 14 showed true positive with one minute rapid urease test. Of these, 12 became positive within one minute and the other two cases became positive within one hour and in 24 hours. Moreover, 21 cases showed weak reactions and these were considered as negative results. A correlation of rapid urease test results with other test methods are is given in Table 13.

Table 13. Modified one minute rapid urease test results in correlation with the other test methods for the detection of *Helicobacter pylori* infection.

Modified one minute RUT +ve = 14	H & E stain +ve	Toluidineblue +ve	IHC +ve	PCR +ve	ELISA +ve	ICT +ve
With 1 min	3	4	1	3	3	2
With 1 hour	1	1	1	1	1	1
In 24 hours	1	1	1	0	1	1
RUT -ve = 191	-	-	-	7	2	10

H & E - haematoxylin and eosin; IHC - immunohistochemistry; RUT - rapid urease test; PCR - polymerase chain reaction; ELISA - enzymelinked immunosorbent assay; ICT - immunochromatography test.

3.6.1 Evaluation of diagnostic efficacy of modified one minute RUT

The sensitivity and specificity of one minute rapid urease test were evaluated against histology, immunohistochemistry and PCR (Table 17)

Table 14. Comparison of modified one minute RUT with histology for the detection of *Helicobacter pylori* infection.

	Histology +ve	Histology -ve	Total
Modified one minute RUT +ve	6	8	14
Modified one minute RUT -ve	0	191	191
Total	6	199	205

Table 15. Comparison of modified one minute RUT with IHC for the detection of *Helicobacter pylori* infection.

	IHC +ve	IHC -ve	Total
Modified one minute RUT +ve	3	11	14
Modified one minute RUT -ve	0	191	191
Total	3	202	205

Table 16. Comparison of modified one minute RUT with PCR for the detection of *Helicobacter pylori* infection.

	PCR +ve	PCR -ve	Total
Modified one minute RUT +ve	11	3	14
Modified one minute RUT -ve	0	191	191
Total	11	194	205

Table 17. Evaluation of diagnostic efficacy of modified one minute RUT for the detection of *Helicobacter pylori* infection.

	Sensitivity	Specificity	Positive predictive value	Negative predictive value
Modified one minute RUT against histology	100%	95.9%	42.8%	100%
Modified one minute RUT against immunohistochemistry	100%	94.5%	21.4%	100%
Modified one minute RUT against PCR	100%	98.4%	78.5%	100%

3.7 Polymerase reaction (PCR) results

Of the 205 patients, only 11 became positive with PCR for the presence of *Helicobacter pylori* infection. The six histology positive patients' samples were retested with PCR.

Table 18. PCR test results in correlation with the other test methods for the detection of *Helicobacter pylori* infection.

PCR	H and E +ve	Toluidine blue +ve	IHC +v	Modified one minute RUT +ve	ELISA +ve	ICT +ve
PCR +ve = 11	3	3	2	4	3	2
PCR-ve = 194	-	-	-	10	4	12

H & E - haematoxylin and eosin; IHC - immunohistochemistry; RUT - rapid urease test; PCR - polymerase chain reaction; ELISA - enzymelinked immunosorbent assay; ICT - immunochromatography test.

3.7.1 Evaluation of diagnostic efficacy of PCR

The sensitivity and specificity of PCR test were evaluated against histology and immunohistochemistry (Table 21).

Table 19. Comparison PCR with histology for the detection of *Helicobacter pylori* infection.

	Histology +ve	Histology -ve	Total
PCR +ve	6	5	11
PCR-ve	0	194	194
Total	6	199	205

Table 20. Comparison PCR with immunohistochemistry for the detection of *Helicobacter pylori* infection.

	IHC +ve	IHC-ve	Total
PCR +ve	3	8	11
PCR-ve	0	194	194
Total	3	202	205

Table 21. Accuracy indices of PCR for the detection of *Helicobacter pylori* infection.

	Sensitivity	Specificity	Positive predictive value	Negative predictive value
PCR against histology	100%	97.4%	54.5%	100%
PCR against IHC	100%	98.4%	27.2%	100%

3.8 Serology results

3.8.1 ELISA for detection of anti-*H.pylori* IgG antibodies

Calculation of ELISA results

Firstly, the mean of the duplicate calibrator value Xc was calculated. Then mean of the duplicate positive control, negative control and the patient samples were calculated.

Finally the anti-*H.pylori* IgG EIA Index of each sample was calculated by dividing the values of each sample Xs by calibrator mean value Xc.

Example shown using Patient No HP 131:

1. Cut off calibrator O.D =1.267, 1.279 Xc =1.273

2. Neagtive control O.D =0.061, 0.066 Xn=0.063

   H. pylori IgG EIA Index = Xn/Xc
   =0.063/1.273
   =0.049

3. Positive control O.D=1.1404, 1.1455 Xp=1.4295

   H.pylori IgG EIA Index =Xp/Xc
   = 1.4295/1.273
   =1.122

4. HP 131sample O.D value Xs =1.432

   H.pylori IgG EIA Index =Xs/Xc
   =1.432/1.273
   =1.124

Interpretataion of ELISA results

Negative: *H.pylori* IgG EIA Index <0.90 - sero negative for anti-*H.pylori* IgG.

Equivocal: *H.pylori* IgG EIA Index between 0.91-0.99 was equivocal.

Positive: *H.pylori* IgG Index of 1.00 or more is sero positive for anti-*H.pylori* IgG.

Thus out of 205 patients, only 7 became positive with ELISA for the presence of anti-*H.pylori* IgG.

Table 22. ELISA results in correlation with the other tests for the detection of *Helicobacter pylori* infection.

ELISA	H and E +ve	Toluidine blue +ve	ICT +ve	IHC +ve	Modified one minute RUT +ve	PCR +ve
7	4	5	6	3	5	3

H & E - haematoxylin and eosin; IHC - immunohistochemistry; RUT - rapid urease test; PCR - polymerase chain reaction; ELISA - enzymelinked immunosorbent assay; ICT - immunochromatography test.

3.8.2 Immunochromatography for the detection of anti-*H.pylori* antibodies

Of the 205 patients, only 14 became positive with immunochromatography for anti-*H.pylori* antibodies.

Table 23. Immunochromatography in correlation with the other test for the detection of *H. pylori* infection.

ICT	H &E +ve	Toluidine blue +ve	IHC +ve	Modified one minute RUT +ve	PCR +ve	ELISA +ve
3 Strong +ve patients	1	1	-	2	-	3
11 weakly +ve patients	3	3	3	3	2	3

The false positive cases for ICT were more, therefore weakly positive cases should considered as negative for ICT.

3.8.3 Evaluation of diagnostic efficacy of immunochromatography test

The sensitivity and specificity of ICT were evaluated against ELISA (Table 25).

Table 24. Comparison of ICT with ELISA for the detection of anti-*Helicobacter pylori* IgG.

	ELISA +ve	ELISA -ve	Total
ICT +ve	7	7	14
ICT -ve	0	191	191
Total	7	198	205

Table 25. Accuracy indices of ICT for the detection of *Helicobacter pylori* infection.

	Sensitivity	Specificity	Positive predictive value	Negative predictive value
ICT against ELISA	100%	96%	50%	100%

3.9 Cost evaluation for modified one minute RUT, ICT, IHC, ELISA and PCR for the detection of *Helicobacter pylori* infection.

Table 26. Cost evaluation of the different test methods used in the study.

Test Method	LKR
IHC	2500
PCR	1500
ELISA	1000
ICT	300
RUT	25

Each test method required different chemical and reagents and thus the cost involved with each detection method varied. The cost of the each test methods is given in the Table 26.IHC and PCR is the most expensive test methods used in the study. Moreover, ICT is the most cost effective when compared to ELISA. Modified one minute RUT is the lowest of the detection method tested in the study.

Chapter 4 -Discussion and conclusions

The review of literature on prevalence of *H.pylori* infection in Sri Lanka using different test methods shows different rates of prevalence ranging from 2.9% to 70.1%. In the present study, the prevalence rates varied with the test method used. However, the results do not show marked variations and the overall prevalence of *H. pylori* infection in the population appear to be relatively low compared to other developing countries and some developed countries. The reason for this observation is not clear. One possible reason might be the antibiotic treatment to other bacterial infections. However, the low sero-prevalence of anti-*H.pylori* IgG indicates that the exposure rates to *H. pylori* in this sample is low, favoring a true low prevalence.

4.1 Histology

Prevalence rate of 2.9% based on histology in the present study was much lower than a prevalence rate of 49.4% reported by Waidyarathne et al (2010). In the current study, both H & E and toluidine blue stains gave similar results. PCR was used as a comparator to compare the histology results. When comparing with immunohistochemistry, histology had 100% sensitivity and 98.5% specificity (Table 7). Although the histology is sensitive in detecting the presence of the *H. pylori*, interpretation depends on the experience of the histopathologist. False positive results are known to occur due to over-interpretation of debris and other coliforms that might be present on the gastric mucuosa as a contaminant from food. Less commonly, other *Helicobacter* species, such as *Helicobacter heilmanii* which can also be detected in the human gastric mucosa can give rise to false positive

results. *H.heilmanii*, a zoonotic infection in humans that can be acquired from cats or dogs and can cause chronic gastritis, is present in about 0.1% of gastric biopsies (Morgner 2000).

Detection of *H. pylori* based on histology had a sensitivity of 54.4% and a specificity of 100% when compared with PCR in the present study (Table 7). When compared with PCR, the sensitivity of histology was relatively low for detecting *H. pylori* infection. The sensitivity and specificity of histology for *H. pylori* detection vary from 53% to 90%, depending on the experience of the histopathologist and the density of colonization. Increasing the number of biopsies and employing specific stains can increase the sensitivity of histology (El-Zimaity 1999). However, in the present study, addition of multiple biopsies did not increase the yield of bacteria and all the positive patients had *H. pylori* in the antral lesion. Furthermore, large majority of histologically negative patients for *H.pylori* did not have any gastric mucosal inflammation. Since PCR is a highly sensitive method it can detect even very low virulent forms of *H. pylori* in small numbers which are not capable of producing gastric inflammation and clinically significant disease. But all histology positive patients except one had some degree of inflammation and others had moderate to severe inflammation.

Furthermore, histology has the advantage of assessing the severity of the disease, the inflammatory response and complications including atrophy dysplasia, intestinal metaplasia and gastric malignancies. Therefore, histology is important in identifying the clinically significant *H .pylori* infection and should remain as a test method in the diagnostic laboratories.

Moreover, in the present study, use of a more sensitive PCR to evaluate the diagnostic efficacy of histology has underestimated its diagnostic values of clinically significant *H. pylori* infection as indicated by very low sensitivity and positive predictive value (PPV). However, histology requires infra structure for tissue processing and experienced histopathologist for the interpretation of histology.

When considering the inflammation detected by histology, significantly large number of cases with *H. pylori* infection had Sydney grade 2 or 3 chronic inflammation. In other words patients with Sydney grade 2 or 3 chronic inflammation had 40% *H. pylori* infection and the likelihood of detection of *H. pylori* appears to increase with increasing severity of chronic inflammation. Also there were only 3 cases with acute inflammation in addition to chronic inflammation. Two had Sydney grade 2 and 1 had Sydney grade 1.Furthermore, even in the case of *H. pylori* infection, the density of the organisms was low in most cases. Reasons for the absence of *H. pylori* infection in the rest of the cohort includes past infection with *H. pylori*, partial treatment with antibiotics which could give rise to very low number of *H. pylori* load causing absence of infection and inflammation. However, in the present study population these reasons are less likely due to low prevalence (3.4%) of anti-*H. pylori* IgG, which indicates a low level of present / past infection, detected by ELISA.

Moreover, in our study, majority of the patients with endoscopically visible gastric inflammation did not show inflammation histologically.

When considering the endoscopic findings, gastropathies were diagnosed in high numbers. Common causes of gastropathies are bile reflux, drugs such as non-steroidal anti-inflammatory drugs and various vascular gastropathies such as portal hypertensive gastropathy (Chandrasoma1999; Day 2003). The 70.9% of the patients that had endoscopic inflammation did not have true histologic inflammation and the reason for this finding might be due to aforementioned gastropathies of which the bile reflux is a common but often undetected condition.

A main limitation in histology, as observed in this study, was less than satisfactory inter-observer agreement, which was 0.4 for H & E and 0.3 for toluidine blue; ideally, it would want an agreement or ICC of 0.8 or above. Over interpretation of results, as indicated by other tests methods used, was the main reason for the limited inter-observer agreement. However, one limitation of the study was participation of only four observers to assess the inter-observer agreement. Disagreement with even one participant can significantly affect the result when numbers of participants are less. Therefore, this should be better assessed using more numbers of observers.

4.2 Immunohistochemistry

Immunohistochemistry (IHC) detected 3 positive cases of *H. pylori* infection. The IHC test had a sensitivity of 27.2% and a specificity of 100% when compared to PCR as a comparator (Table 11). The IHC positive patients were positive by all the other test methods except PCR and only 2 IHC test positive patients were positive for PCR.

IHC is considered as the highly sensitive method for identifying *H. pylori* in gastric biopsies. It is more sensitive than other conventional stains and considerably easier to interpret. However, our study found a *H. pylori* positivity of 2.9% for H & E and toluidine blue staining methods when compared to IHC which showed only 1.4% positivity for *H. pylori* (Table 3). Conversely, with H & E curved bacteria are only detected when found in large numbers. Using IHC with specific antibodies, it is possible to identify *H. pylori* even in small numbers. However, IHC stain would have missed the *H. pylori* in the gastric mucosa due to the specific monoclonal antibodies targeting only the *H. pylori* but not all curved bacteria which are targeted by H & E and toluidine blue staining methods (Genta 1994). Because of the high cost per case and relatively low sensitivity makes IHC less useful for routine diagnosis of *H. pylori* infection in clinical samples from patients with possible *H. pylori* gastritis.

4.3 Modified one minute rapid urease test

The *H. pylori* prevalence of modified one minute RUT in our study was 6.8% which lies between the prevalence rates resulted from the previous studies by Fernando et al (1992) of 60% and De Silva et al (1999) of 2.9% using CLO test. The sensitivity of modified one minute RUT when compared with PCR, immunohistochemistry and histology was 100% and specificity was 98.4%, 94.5% and 95.9%, respectively (Table 16). However, the main limitation in the current study was presence of more false positives of modified one minute RUT and thus resulting in a low PPV and this could be due to low *H. pylori* prevalence in the sample.

Superior sensitivity and the NPV of modified one minute rapid urease test are due to observation of the test up to 24 hours. The two late positives were true positives. Therefore, we recommend observing the test solution with biopsy tissue up to 24 hours as in CLO test. Furthermore, absence of true positives in the 21 cases with weak colour changes justifies treating weak reactions as negative. Interpretation of weak colour changes as positive would have reduced the specificity of the test.

It is noteworthy that the late reacting case which became positive within 24 hours showed only subtle chronic inflammation with no active inflammation despite heavy colonization with *H. pylori*. Positive result with *H. pylori* specific IHC confirmed the presence of *H. pylori*. Of the modified one minute RUT positive patients, only 4 were positive for PCR. *H. pylori* is known for genetic polymorphism and some strains are known to be less virulent than the others, for example, the vacA s2/m2 strains are shown to be virtually non-toxic. Therefore, it is possible that this particular patient was infected with a less virulent *H. pylori* strain as supported by the findings of previous studies conducted elsewhere (Yamaoka 2010; Atherton 1995).

There are many causes for false positive results in modified one minute RUT. Patients who are on long term proton pump inhibitors may develop achlorhydria with subsequent superficial colonization of the gastric mucus layer with non-*H. pylori* urease producing organisms like *Protease mirabilis* or *Klebsiella*. However, *H. pylori* urease is much more potent than the urease produced by other organisms and thus they tend to give delayed results after 24 hours of inoculation and generally negative at one hour (Midolo 2000; Mobley 1988).

The non-*H.pylori* bacteria such as *Helicobacter heilmanni* which infect gastric mucosa also give rise to false positive results by modified one minute RUT. However, *H. heilmanni* accounts for about 1% of gastritis and usually gives rise to a weak reaction (Midolo 2000).

The main limitation of the study is the low prevalence of *H. pylori* infection in the sample tested (2.9%) which can influence the predictive values of the detection methods. This could be the reason for the low PPV values of the present test. The low sero-prevalence of anti-*H.pylori* antibodies (3.41%) confirms that the *H. pylori* prevalence in the sample is truly low and the study sample had a low exposure rate to the *H.pylori*. In contrast, one minute RUT by Arvind et Al (1988) showed a PPV of 100% when the disease prevalence in the sample was 52.5%.

Although the urea solution should ideally be prepared fresh in the original study, researchers of another previous study had observed that pre-prepared frozen solutions could also be used by thawing just before use (Chey 2007). In our experience, storage of pre-prepared urea solution in a domestic type refrigerator up to 7 days did not significantly change the test efficacy. The optimal action of urease enzyme is at 45 $^{\circ}C$ and therefore, it is important to bring the solution to room temperature before testing to prevent false negatives. The high sensitivity, specificity and NPV, low cost and simple methodology were the advantages of the modified one minute RUT and the main limitation of the test was low PPV. Hence, modified one minute RUT can be considered as a suitable low cost method to detect *H. pylori* infection in gastric biopsies in resource limited settings.

4.4 PCR

PCR is regarded as a highly sensitive and specific test for detecting *H. pylori* in research studies. We have used a PCR as a standard comparator in the study. The sensitivity and specificity of the PCR with IHC were 100%, 97.4%, respectively and these indices with histology were 100%, 98.4%, respectively (Table 20).

PCR prevalence rate of 5.3% for *H. pylori* in the present study is lower than the prevalence rate (70.1%) reported by Fernando et al (2002) using a sample of dyspeptic patients. One difference between the two studies is that the current study has tested only one DNA locus (16SrRNA) and Fernando et al (2002) had tested for two DNA loci, 16SrRNA and ureC (glmM). Target locus 16SrRNA is widely used in PCR of *H. pylori* and 16SrRNA has been proven to have a higher sensitivity (Ho 1991, Lu 1999), whereas ureC (glmM) has a comparatively lesser sensitivity and higher specificity (Lu 1999). Hence, combination of 16SrRNA and ureC (glmM) is a better choice as used in Fernando et al (2002), however, does not explain the marked difference in results observed in the two studies. Since we have used primers targeting 16SrRNA as used by others it is less likely that lower rates of *H. pylori* observed in the study sample is due false negatives. Although, DNA degradation could occur with adverse transport and storage, PCR is less affected by such factors compared to other tests, even presence of a small amount of specific DNA is sufficient for the positive test (Ho 2000). Furthermore, we have immediately frozen the biopsy and stored -80°C and thawed only at the time of extraction of DNA. Accordingly, loss of DNA during transport and storage is less likely in our study. Patchy distribution of the infection in the gastric mucosa is also a reason for false negative PCR (Ho 2000).

Although we have used only one biopsy from the inflamed areas from the antrum, we have extensively biopsied the entire gastric mucosa and ruled out the presence of inflammation in the rest of the mucosa histologically. It is extremely rare to get clinically significant *H. pylori* infection without inflammation. Low prevalence rates for *H.pylori* by histology and serology support that PCR has not produced false negative results in our study. Virulence of *H. pylori* is determined by its ability to adhere to the gastric surface epithelium and induce an inflammatory response in the gastric mucosa (Yamaoka 2010). In the present study, although PCR has shown the highest *H. pylori* positive rates with 11 patients positive for PCR alone, only 4 patients had inflammation and of these 4 only one had severe inflammation, 2 had moderate inflammation and one had mild inflammation. Absence of significant inflammation in the only PCR positive group indicates that the positive results are probably not clinically significant and symptoms in the patients may not be due to *H. pylori* infection. PCR is a highly sensitive method in detecting *H. pylori* DNA and presence of 10 to 100 *H. pylori* per sample is adequate to give positive results (Ho 2000). It is possible that in this study, only PCR positive patients may have less virulent strains of *H. pylori* present in low numbers, not able to establish gastric inflammation. Furthermore, such high sensitivity increases the risk of false positive results and even minor contamination can give rise to false positive results. Therefore, PCR may not be suitable for routine diagnosis of *H. pylori* infection and may over-diagnose the disease. Requirement of infrastructure and high cost of the test also do not favour the utility of PCR in a routine diagnostic laboratory.

4.5 Serology

The two main testing methods used in serology are ELISA and ICT. ELISA is the recommended test method by American Gastroenterology Association and Maastricht/Florence Consensus report for the exposure to *H. pylori* (Europe) (Chey 2007; Malfertheiner 2012) because of its higher diagnostic efficacy indicators. Both ELISA (3.41%) and ICT (6.82%) methods gave low anti *H.pylori* antibody sero-prevalence rates in the present study population. Although ELISA has higher sensitivity and specificity, it needs laboratory infrastructure, expertise with long turnaround time for test results and higher cost for reagent kits. On the other hand, ICT assay is a rapid test and this test unlike ELISA does not requires pre-treatment of sera, elaborate equipment and can be performed in a matter of minutes. ICT could well be most useful for clinical laboratories performing tests for where immediate results are required for management of patients with the test utility for a single patient when compared to ELISA which needs a batch of samples to be tested. ICT is much cheaper (LKR 300 per patient) than ELISA (LKR 1000 per patient). ELISA specifically look for anti-*H.pylori* IgG antibodies, whereas, ICT methods collectively detects all types of anti *H.pylori* antibodies. *H. pylori* infection produces a very short lived IgM response which has a very limited clinical value. Hence the main limitation with serological diagnosis of *H. pylori* infection is that it cannot differentiate present infection from past infection and cannot be utilized to assess response to therapy. Nevertheless serology is a useful minimally invasive method which indicates the exposure to the infection. Although ELISA is the recommended method for serological diagnosis of *H. pylori* infection, it is not widely used in Sri Lanka due to the cost and ICT is used in the private sector laboratories.

In our study, when comparing ICT with ELISA the sensitivity and specificity were 100% and 96 %.The high sensitivity of ELISA test could be a reason that, *H. pylori* shares similar antigens with other bacterial species such as, as *Campylobacter jejuni* (Perez 1987; Rathbone 1986) and there are differences in the antigenicity of the multiple *H. pylori* strains and even among different antigens of the same strain. (Khaled Abdulqawi 2012). Different component of *H. pylori* such as urease enzyme or high molecular weight cell associated proteins and 25kDa antigen of *H. pylori* also triggers the antibody response with high sensitivity and specificity (Sugiyama 1991). Franceshi et al has demonstrated that there can be human antigens which are similar to the *H. pylori* Cage antibody. These can lead to false positive serological results when compared with other tests that detect *H. pylori* antigens (IHC) or DNA (PCR) (Francaschi 2000).

Histological abnormalities in some patients with sero-positivity for *H. pylori* in the absence of *H. pylori* in the gastric biopsy specimens suggest that they had been infected with *H. pylori* some time in their life (Taha 1993). Numbers of healthy people previously infected with *H. pylori* might remain sero-positive even after the eradication of bacteria (Meyer 1991). Two patients showed positive for anti-*H.pylori* IgG but did not have presence of the *H. pylori* in our study. Moreover, serological studies states that in *H. pylori* infected patients, specific IgA and IgG antibody value were low but not yet returned to the normal value, even after 12 months of anti-*H. pylori* therapy (Veeneda 1991), therefore it is clear that past exposure, as marked by IgG antibody against *H. pylori* will be more in patients infected in the past than those having the active infection.

Immune response to *H. pylori* also can be influenced by different geographical locations due to genetic difference in the populations. However, our results show only a prevalence of 3.41%, indicating a low immune response to *H. pylori* infection when compared with the study carried out by Fernando et al (2003) showing a sero-prevalence of 10.3% for IgG antibody against *H. pylori* and this indicates that the prevalence of *H. pylori* infection has been relatively low in Sri Lanka. Also immune response varies with age, primary infection occur mostly in infants and young children who are expected to have a shorter duration of infection than older children and adults (Mohammed 2008). However, the present study included only adults and thus the study population might have infection with *H. pylori* at an early age. Anti-*H.pylori* IgG, IgM and IgA were detected by ICT and Anti-*H.pylori* IgG was detected by ELISA. There were 14 positive patients for ICT and only 7 patients positive for ELISA and 6 patients positive for both the test methods. The IgM response in *H. pylori* is very short lived to be of any clinical value. Furthermore, unlike in acute infections fourfold rise in antibody titres is not useful in chronic infections to indicate an active infection. Accordingly, the main limitation with serological testing is that it cannot differentiate current *H. pylori* infection from the past exposure and therefore both American Gastroenterology Association and Maastricht IV/Florence consensus reports do not recommend serology to monitor the response to therapy (Chey 2007; Malfertheiner 2012).

4.6 Conclusions

Although the *H.pylori* prevalence rates varied with the test method used from 1.4% with immunohistochemistry, 6.8% with immunochromatography and modified one minute RUT, the overall *H. pylori* prevalence with all test methods used were consistently low, indicating a true low prevalence of *H. pylori* infection in the study population. Low prevalence of serum anti-*H.pylori* IgG levels also suggested a low exposure rate to *H. pylori* infection supporting the low prevalence of *H. pylori* infection in the study cohort.

Modified one minute RUT has been shown to be an inexpensive test with reasonable diagnostic efficacy that can be considered as a cost effective *H. pylori* detection method to be adopted in resource limited settings when other testing methods are not available or as an adjunct to histology.

Although PCR had the highest detection rate for *H. pylori*, the clinical relevance of the test is questionable as most of the patients positive for PCR did not have gastric inflammation. Although the detection rate by histology was comparatively low, most of the histology positive cases had significant gastric inflammation and thus the histology appear to be identifying the cases with clinically significant gastric disease with or without *H. pylori* infection.

ICT shown to have good diagnostic efficacy and it is a low cost serological test alternative for ELISA. Moreover, the main limitation with serological testing is their inability to differentiate current *H. pylori* infection from the past exposure.

Appendices

Appendix 1

Clinical Information sheet **HP Lab**

Reference:

(Helicobacter pylori Study)

Personal Information

Name: ....
......

Age: .... years

Sex...

BHT: ....

Address: ....

Symptoms:

Post prandial fullness	☐	Acid bash	☐
Early satiety	☐	Regurgitation	☐
Epigastric pain or burning	☐	Haematemesis	☐
Retrosternal pain	☐	Melena	☐

Other:..
....

Indication/s for UGIE: ....
......

Prior treatment with triple therapy: ....

If Yes Duration: .... days When: ....

Last time patient was treated with an antibiotic (if known the name of the drug): ....
....

(If patient do not know about the type of drug he/she's been taking state as "do not know")

Endoscopic findings:

Ulcers (specify site):...

Erosions (Specify site):
....

Inflammation(specifysite):...

Other

Body:...

Antrum:..

Duodenum:..

Pylorus: Open: .... Closed:................................

Bile reflux: seen:......................... Not Seen:..................................

Biopsies taken

Lesion/ulcer: .... (3) 2 in formalin, 1 in normal saline

Antrum (outside lesion/ ulcer): ....(1) In formalin

Incisura angularis: .... (1) In formalin

Body:..(1) In formalin

Duodenum (1st part): ....(1) In formalin

Antral biopsy for PCR (fresh): ....

Blood withdrawn for serology: ....

One minute Rapid urease test: Positive ☐ Negative ☐

Appendix 2

Consent form for collecting gastric biopsy for *H. pylori* detection in the Gastric mucosa

I am Dr Sulochana Wijetunge working in the Medical faculty of University of Peradeniya as a senior Lecturer, and Mrs Sabrina Marsha (research assistant).We are doing a research on prevalence of *Helicobacter pylori* and evaluation of different diagnostic tests for detecting *Helicobacter pylori* in symptomatic patients in Sri Lanka. This bacterium is a common cause of producing gastritis. I am going to give you information and invite you to be part of this research.

Purpose of the research
Gastritis is a common disease. *Helicobacter pylori* organism has been recognized as a common cause of gastritis. The purpose of this study is to identify exposure rate of people to this organism and to evaluation of different diagnostic tests for detecting *Helicobacter pylori* in symptomatic patients in Sri Lanka

Procedures and Protocol
Gastric biopsies will be collected according to a protocol which included four biopsies from the antrum and one each from incisura angularis and the body. We will obtain a 2 ml blood sample will from you for the study using a syringe. The blood will be obtained from a superficial vein by a skilled person who has an MBBS degree. The personal details we collect (name and the address) will not be used for the study. Also a questionnaire will be given to you. Data analysis will be performed using a reference number. The biopsy test results report will be issued to you.

Risks: This is a minimally invasive procedure with minimal risks. The two possible risks are bleeding from the site which can be prevented by applying pressure on the site after the needle is withdrawn and the possibility of introducing infection. The latter risk is minimized by adherence to proper aseptic techniques.

Benefits: you will get a report of the test results. Although you will not get much direct benefits from this test, you will contribute to the enhancement of scientific knowledge of the disease gastritis and improve their management in Sri Lanka.

Confidentiality: The information that we collect from this research project will be kept confidential. Information about you that will be collected during the research will be put away and no-one but the researchers will be able to see it. Any information about you will have a number on it instead of your name. Only the researchers will know what your number is and we will lock that information up with a lock and key. It will not be shared with or given to anyone except Dr Sulochana Wijetunge.

Right to Refuse or Withdraw: You do not have to take part in this research if you do not wish to do so and refusing to participate will not affect you in any way. You may stop participating in the research at any time that you wish without losing any of your rights as a patient here.

Duration: We will perform this sample collection today only.

Who to Contact:

If you have any questions you may ask them now or later, even after the study has started. If you wish to ask questions later, you may contact any of the following:

Name:- Dr S. Wijetunge , Mrs. Sabrina Marsha
Address:- Department of Pathology, Faculty of Medicine, University of Peradeniya
Telephone number 0812396648

PART II

Certificate of Consent:

I have read the foregoing information, or it has been read to me. I have had the opportunity to ask questions about it and any questions that I have asked have been answered to my satisfaction. I consent voluntarily to participate as a participant in this research.

Name of Participant__________________
Signature of Participant ___________________
Date ____________________________Day/month/year

If illiterate:

A literate witness must sign (if possible, this person should be selected by the participant and should have no connection to the research team). Participants who are illiterate should include their thumb-print as well.

I have witnessed the accurate reading of the consent form to the potential participant, and the individual has had the opportunity to ask questions. I confirm that the individual has given consent freely.

Print name of witness_____________________Thumb print of participant
Signature of witness ______________________
Date _______________________
Day/month/year

Appendix 3

Reporting Sheet According to Sydney Grade System

Lesion

CI.................. AI........... Atrophy.....................

Intestinal metaplasia .... Dysplasia.....................

HP.......... TB.......... IHC ....

Antrum

CI.................. AI........... Atrophy.....................

Intestinal metaplasia .... Dysplasia.....................

HP.......... TB.......... IHC ....

Body

CI.................. AI........... Atrophy.....................

Intestinal metaplasia .... Dysplasia.....................

HP.......... TB.......... IHC ....

Incisura angularis

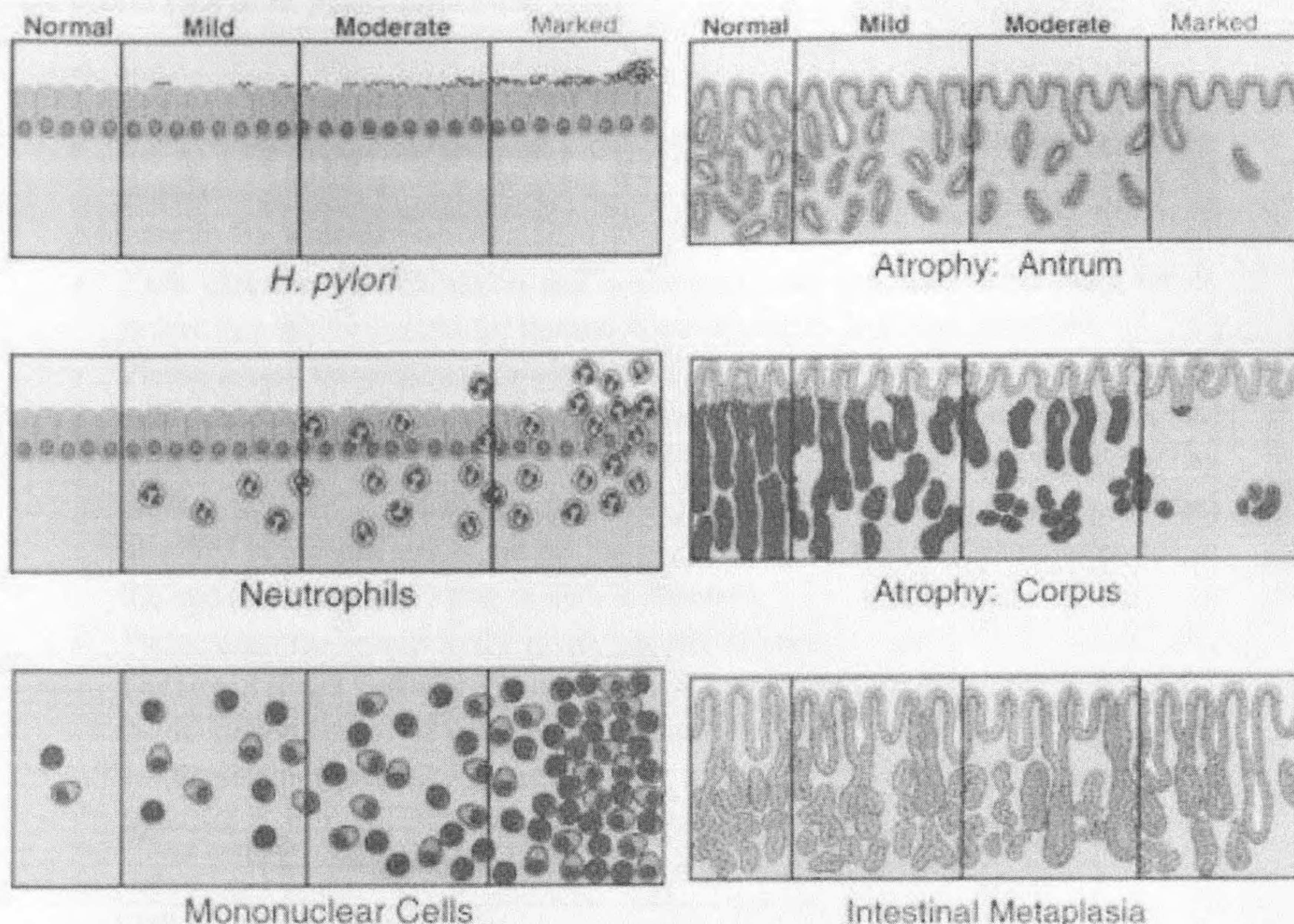
CI.................. AI........... Atrophy.....................

Intestinal metaplasia .... Dysplasia.....................

HP.......... TB.......... IHC ....

Appendix 4

Visual analogue scales for Grading of chronic gastritis according to the updated Sydney grading system



Using the visual analogue scales: The observer should attempt to evaluate one feature at the time. The most prevalent appearance on each side should be matched with the graded panel that resembles it most closely. Observers should keep in mind that these drawings are not intended to represent realistically the histopathological appearance of the gastric mucosa; rather, they provide a schematic representation of the magnitude of each feature and, as such, have certain limitations. Thus, for example, the decreasing thickness of the mucosa usually observed with increasing atrophy is not depicted realistically. Particularly with *Helicobacter pylori* and neutrophils, there may be a considerable variation of intensity within the same biopsy sample; in such cases, the observer should attempt to average the different areas and score the specimen accordingly.

From: Dixon MF et al. Classification and grading of gastritis: The updated Sydney system. American Journal of Surgical Pathology 1996: 20; 10: 1161 – 1181.

Appendix 5

Assessment of inter-observer variations

Detection rate of *H. pylori* infection:

Instructions:

- Each case contains histological sections from biopsies from four regions in the stomach:

A) Lesion; B) Antrum

- Each case has Haematoxylin and eosin stain and Toluidine blue stain for *H. pylori.* A positive control for immunohistochemistry has been provided.
- Please assess the presence or absence of *H. pylori* organisms by Haematoxylin and eosin stain and, toluidine blue stain. If organisms are present grade the density of the organisms in H and E stain and toluidine blue stain using Sydney grading system on a scale grade 1(mild) to grade 3 (severe). Grading of other histological parameters is not necessary. The visual analogue scale provided in the updated Sydney grading system is attached.
- Please enter the results in the given table as follows:
  For H and E and Toluidine blue stains
  0 – no organisms 2 – present moderate
  1 – Present mild 3 – present severe

Case number	H and E	Toluidine blue
HP 1		
HP 2		
HP 3		
HP 4		
HP 5		
HP 6		
HP 7		
HP 8		
HP 9		
HP10		
HP11		
HP12		
HP13		

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