CHAPTER-I

1. INTRODUCTION

The discovery of *Helicobacter pylori*, a gram negative spiral shaped, microaerophilic bacterium, by Warren and Marshall not only introduce a whole new group of bacteria to science but also revolutionized the concept of gastroduodenal pathology and diverted the worldwide attention from pH to Hp (Arora *et al.*, 2003).

H. pylori is the only known organism that can thrive in the highly acidic environment of stomach lining representing a key factor in the etiology of various gastrointestinal diseases, ranging from chronic active gastritis without clinical symptoms to peptic ulceration, gastric adenocarcinoma, one of the most common cause of cancer death in the world, gastric non-hodgkin's lymphoma, gastric mucosa-associated lymphoid tissue (MALT) lymphoma (MALToma), B-cell mucosa-associated lymphoid tissue (MALT) lymphoma and menetrier's disease.

H. pylori is the first formally recognized bacterial carcinogen and is one of the most successful human pathogens, as over half of the world's population is colonized with this bacterium (Kusters *et al.,* 2006), 30 to 50 % of adults in developed countries while almost universal in developing countries. Colonization increases progressively with age, and children are believed to be the major amplifiers of *H. pylori* in human population (Kenneth *et al.,* 2004). Unless treated, colonization usually persists lifelong. The prevalence of the infection is greater in developing countries and is influenced by socioeconomic conditions, ethnic background and age (Destura *et al.*, 2004).

Currently, there are several popular methods for detecting the presence of *H. pylori* infection, each having its own advantages, disadvantages, and limitations (Bravos and Gilman, 2000). They may broadly be divided into tests that indirectly determine the presence of the microorganism (antibody tests in blood, urine, or saliva) or direct tests that detect the intact organism (histology and culture), antigens shed from the organism (stool antigen test), or metabolic functions of the organism (rapid urease test and urea breath test) (Holton *et al.,* 2000). The choice of a specific test for an individual patient depends on local experience and the clinical setting (Logan, 1998, Vaira *et al.,* 1999, Zagari *et al.,* 1999). For routine

diagnostic purposes, histology, urea breath testing, and culture are currently most often used, whereas the use of serology is most appropriate for large epidemiological studies. In hospital-based care, many patients undergo endoscopy, which is then combined with an invasive test for *H. pylori*. Otherwise, breath tests and serology are commonly used. For children, fecal antigen tests offer the opportunity to assess *H. pylori* status without the need for endoscopy or vena puncture (Kusters *et al.,* 2006).

The current recommended first line treatment for *H. pylori* infection is a triple therapy that includes a proton pump inhibitor or ranitidine bismuth citrate with two broad-spectrum antibiotics, usually from clarithromycin, amoxicillin, or metronidazole (Malfertheiner *et al.,* 2000). The Maastricht consensus report recommends starting with clarithromycin triple therapy, following with quadruple therapy in the case of failure, and using levofloxacin triple therapy (consisting of a proton-pump inhibitor, amoxicillin, and levofloxacin) if the first two strategies fail (Malfertheiner *et al.,* 2007) . With this approach, a 90% overall eradication rate can be achieved (Rokkas *et al.,* 2009). In contrast, the triple-therapy regimen suggested by McColl (consisting of a proton pump inhibitor, amoxicillin, and metronidazole) had a poor eradication rate, 76% (95% confidence interval, 68 to 84), and is a less satisfactory alternative (Bardhan *et al.,* 2000)

Drawbacks of current therapies include inadequate drug delivery, lack of patient compliance, the development of antibiotic-resistant *H. pylori* strains, and the adverse side-effects associated with eradication of beneficial gastrointestinal flora through the use of broad spectrum antibiotics (Duckworth *et al.,* 2006).

Antibiotic resistance has increasingly been recognized as a major cause of treatment failure for *H. pylori* infection. Primary antimicrobial resistance against clarithromycin and metronidazole is now commonplace in several countries (Poon *et al.,* 2002, Kato *et al.,* 2002, Kim *et al.,* 2001, Meyer *et al.,* 2002, Miyagi *et al.,* 1997, Pilloto *et al.,* 2000, Wolle *et al.,* 2002, Vicente *et al.,* 2002). Regional variations in susceptibility and resistance patterns may be ascribed to differences in local antibiotic prescription practices, antibiotic usage in the community and mass eradication programs for *H. pylori* infection as part of gastric cancer prevention strategies. These factors may well be expected to influence success of eradication therapy (Wong *et al.,* 2002, Lui *et al.,* 2003, A B, 1993, ER D, 1997).

Procuring the fourth position (3.39%) among the top ten OPD diseases, gastritis conveys its scenario in Nepal and its frequency is found to be greater in mountainous region than in Hilly and Terai region. Peptic ulcer and gastric cancer cases have also been inspected in the same report (DoHS, 2008/2009). Information regarding the prevalence of *H. pylori* is truly restricted in Nepal. However, different subpopulation studies have been undertaken.

In Nepal, histopathology is unquestionably the first and foremost choice for the diagnosis of *H. pylori* infection. Nevertheless rapid urease test is also in application (Rai *et al.,* 2006; Makaju *et al.,* 2006). Very few seroprevalence studies have also been reported (Kawasaki *et al.,* 1998)

Realizing the increasing prevalence of antimicrobial resistance in other countries and its potential negative impact on the efficacy of many treatment eradication regimens, it is important in clinical practice to determine the prevailing local antibiotic susceptibility patterns when choosing appropriate eradication regimens for *H. pylori* infections in the empiric setting (Destura *et al.,* 2004). Unfortunately, authentic data pertaining to this global issue is still countable in Nepal.

With the goal of attaining the prevalence of *H. pylori* infections and antibiotic resistance pattern among *H. pylori* isolates in Nepal, this study is executed in the patients with gastroduodenal diseases visiting Tribhuvan University Teaching Hospital.

CHAPTER-II

2. OBJECTIVES

2.1 General Objective

To determine the prevalence of *H. pylori* infections and assess antibiotics susceptibility pattern among the patients attending endoscopy unit, TUTH.

2.2 Specific Objectives

1. To compare efficacy of serology as a diagnostic test with respect to rapid urease test, histology and culture.

CHAPTER-III

3. LITERATURE REVIEW

3.1 History

In the year 1875 G. Bottcher in collaboration with M. Letulle, after finding bacteria colonizing gastric ulcers (GUs) and ulcer margins, regarded the bacteria responsible for the ulcer (Kidd and Modlin, 1998). Then in 1889 W. Jarowski found spiral organisms in gastric washings, named them *Vibrio rugula* and suggested they were pathogenic in gastric diseases (Konturek, 2003). In 1896 H Salomon could transfer the infection to mice, and 10 years later W. Krieniz associated the bacteria with gastric cancer. J.M. Luck discovered gastric mucosal urease as early as 1924. Investigations continued and in 1940 F.D. Gorham postulated gastric acidophilic bacteria as an aetiologic agent in PUD and treated PUs with bismuth (Kidd and Modlin, 1998). However, E.D. Palmer later could not find these bacteria in vacuum biopsies in 1088 patients, and the issue was forgotten for 30 years (Palmer, 1954). Finally, Robin Warren in 1979 rediscovered the bacterium, and he with Barry Marshall, over 100 years after the first description, cultured it and by Koch's postulates proved it to be the cause of gastritis and subsequently of PU in1982 (Marshall and Warren, 1984). This was the beginning of a new era in gastroduodenal diseases.

After isolation and identification in 1983, it was first named *Campylobacter pyloridis* due to its location and the resemblance to *Campylobacter* which was later changed to *Campylobacter pylori*. Again it was found that the bacterium differed from *Campylobacter* in important features such as flagellum morphology, fatty acid content and 16S rRNA sequence (Andersen and Wadstrom, 2001) and the name was changed to *Helicobacter pylori*, as it represented its own genus (Goodwin *et al*, 1989). *Helicobacter* reflects the two morphological forms of the organism, helical *in vivo* but often rod-like *in vitro.*

After its discovery, numerous research groups verified the association of *H. pylori* with gastritis and ulcers. In 1994, the National Institutes of Health (USA) published a view stating that most recurrent duodenal and gastric ulcers were caused by *H. pylori* and recommended that antibiotics be included in the treatment regimen (NIH Consensus Statement). Warren and Marshall were jointly awarded the 2005 Nobel Prize in Physiology or Medicine for their

discovery of “the bacterium *Helicobacter pylori* and its role in gastritis and peptic ulcer disease” (The 2005 Nobel Prize in Physiology or Medicine).

3.2 Microbiology

Kingdom: Bacteria
Phylum: Proteobacteria
Class: Epsilon
Proteobacteria
Order: Campylobacterales
Family: Helicobacteraceae
Genus: Helicobacter
Species: *H. pylori*
(Goodwin *et al*, 1989)

3.2.1 Genome, plasmids, and strain diversity

The size of the two sequenced *H. pylori* genomes is 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 J 99 includes only 1,491 genes (Alm *et al.,* 1999, Boneca *et al.,* 2003, Tomb *et al.,* 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 and Hass, 1995). Some of these plasmids form the basis of *H. pylori-E. coli* shuttle vectors used in molecular cloning experiments (Heuermann and Hass, 1998). The existence of *H. pylori*-infecting bacteriophages has been reported, but detailed characterization is lacking (Schmid *et al.*,1990).

In contrast to other bacterial pathogens that are highly clonal (such as *Shigella dysenteriae* and *Mycobacterium tuberculosis*), *H. pylori* is genetically heterogeneous, suggesting a lack of clonality. This results in every *H. pylori*-positive subject carrying a distinct strain (Kansau *et al.,* 1996), although differences within relatives may be small.
The genetic heterogeneity is possibly an adaptation of *H. pylori* to the gastric conditions of its host, as well as to the distinct patterns of the host-mediated immune response to *H. pylori* infection (Kuipers *et al.,* 2000)

3.2.2 Morphology

H. pylori is a gram-negative bacterium, measuring 2 to 4 μm in length and 0.5 to 1 μm in width. Although usually spiral-shaped, the bacterium can appear as a rod, while coccoid shapes appear after prolonged in vitro culture or antibiotic treatment (Kusters *et al.,* 1997). These coccoids cannot be cultured in vitro and are thought to represent dead cells (Kusters *et al.,* 1997), although it has been suggested that coccoid forms may represent a viable, nonculturable state (Enroth *et al.,* 1999). The organism has 2 to 6 unipolar, sheathed flagella of approximately 3 μm in length, which often carry a distinctive bulb at the end (O'Toole *et al.,* 2000). The flagella confer motility and allow rapid movement in viscous solutions such as the mucus layer overlying the gastric epithelial cells (O'Toole *et al.,* 2000).

3.2.3 Growth requirements

A key feature of *H. pylori* is its microaerophilicity, with optimal growth at oxygen levels of 2 to 5% and the additional need of 5 to 10% carbondioxide and high humidity. There is no need for hydrogen, although it is not detrimental to growth. Many laboratories utilize standard microaerobic conditions of 85% nitrogen, 10% carbondioxide, and 5% oxygen for *H. pylori* culture. Growth occurs at 34 to 40°C, with an optimum of 37°C. Although its natural habitat is the acidic gastric mucosa, *H. pylori* is considered to be a neutralophile. The bacterium will survive brief exposure to pHs of < 4, but growth occurs only at the relatively narrow pH range of 5.5 to 8.0, with optimal growth at neutral pH (Scott *et al.,* 2002, Stingl *et al.,* 2002).

H. pylori is a fastidious microorganism and requires complex growth media. Often these media are supplemented with blood or serum. These supplements may act as additional sources of nutrients and possibly also protect against the toxic effects of long-chain fatty acids. The latter function may also be performed by more defined medium supplements, such as β-cyclodextrins or IsoVitaleX, or by using activated charcoal (Taneera *et al.,* 2002). Commonly used solid media for routine isolation and culture of *H. pylori* consist of Columbia or brucella agar supplemented with either (lysed) horse or sheep blood or, alternatively, newborn or fetal calf serum. For primary isolation but also routine culture, selective antibiotic mixtures are available such as vancomycin, trimethoprim, polymyxin B, and amphotericin B (Skirrow,1977). Liquid media usually consist of either brucella, Mueller-Hinton, or brain heart infusion broth supplemented with 2 to 10% calf serum (Reynolds and Penn, 1994).

Isolation of *H. pylori* from gastric biopsy samples is difficult and not always successful. Cultures should be inspected from day 3 to day 14. *H. pylori* forms small, translucent, smooth colonies (Han *et al.,* 1995). Upon successful subculturing *H. pylori* isolates tend to adapt to the growth conditions used in the laboratory. Subsequently, good growth can generally be achieved following 1 to 3 days of incubation when reference strains and laboratory-adapted isolates of *H. pylori* are used. It should be noted that once a culture reaches the stationary phase, the growth rate rapidly declines, accompanied by the morphological change to a coccoid form (Kusters *et al.,* 1997). Prolonged culture does not lead to any significant increase in colony size but rather leads to a transition to the unculturable coccoid state.

3.2.4 Metabolism

H. pylori exhibits a narrow host and target organ range, but infection is usually lifelong. This suggests strong adaptation to its natural habitat, the mucus layer overlying the gastric epithelial cells. As a consequence, *H. pylori* lacks several of the biosynthetic pathways commonly found in less specialized bacteria, such as many enteric bacteria (Alm *et al.,* 1999, Berg *et al.,* 1997, Doig *et al.,* 1999 , Marais *et al.,* 1999, Tomb *et al.,* 1997).

H. pylori is urease, catalase, and oxidase positive, characteristics which are often used in identification of *H. pylori*. *H. pylori* can catabolize glucose, and both genomic and biochemical information indicates that other sugars cannot be catabolized by *H. pylori* (Berg *et al.,* 1997, Doig *et al.,* 1999, Marais *et al.,* 1999, Nedenskov 1994).

Metabolic systems involved in or connected to the virulence of *H. pylori*;

I. Respiration and oxidative stress defense

H. pylori is a microaerophilic bacterium that does not tolerate high oxygen conditions, but it requires at least 2% O_2 (Mendz *et al.,* 1997). This is because *H. pylori* uses oxygen as a terminal electron acceptor. *H. pylori* cannot utilize alternative electron acceptors, such as nitrate or formate, although there is a single report on anaerobic growth of *H. pylori* using fumarate (Smith and Edwards, 1995). In the human host, *H. pylori* is thought to be exposed to oxidative stress produced by the active immune response. To combat such forms of oxidative stress, *H. pylori* expresses several key components of bacterial oxidative stress resistance; these include the superoxide stress defense mediated via the iron-cofactored superoxide dismutase (SodB) (Barnard *et al.,* 2004, Enroth and Engstrand, 1995, Seyler *et al.,* 2001) and

the peroxide stress defense mediated via catalase (KatA) and alkyl hydroperoxide reductase (AhpC) (Harris *et al.*, 2002, Olczak *et al.*, 2003).

II. Nitrogen metabolism

Amino acids and urea are the two major sources of nitrogen in the gastric environment. Since ammonia is a key component in nitrogen metabolism as well as acid resistance (Stingl *et al.*, 2002), it is not surprising that *H. pylori* can utilize several alternative sources of ammonia (Bury-Mone *et al.*, 2003, McGee *et al.*, 1999,Merrell *et al.*, 2003, Skouloubris *et al.*, 2001, Skouloubris *et al.*, 1997, Van Vliet *et al.*, 2003). The different pathways contributing to ammonia synthesis are regulated in response to different stimuli, which probably allows *H. pylori* to switch different pathways on or off depending on the environmental conditions.

III. Metal metabolism

Metals are cofactors of enzymes, catalyzing basic functions such as electron transport, redox reactions, and energy metabolism, and are essential for maintaining the osmotic pressure of the cell. Since both metal limitation and metal overload delay growth and can cause cell death, metal homeostasis is of critical importance to all living organisms.

A. Nickel

H. pylori requires efficient acquisition of nickel, as this is the metal cofactor of the essential colonization factors urease and hydrogenase. Nickel availability in human serum is very low (2 to 11 nM), and the nickel concentration in ingested food varies significantly depending on the diet and on food sources (Christensen *et al.*, 1999, Sunderman *et al.*, 1989)

B. Iron

In tissues of human or animal hosts, the concentration of free iron is too low to support bacterial growth, as most iron is complexed into hemoglobin or chelated by transferrin in serum or by lactoferrin at mucosal surfaces (Van Vliet *et al.*, 2001). Iron sources available in the gastric mucosa are lactoferrin, heme compounds released from damaged tissues, and iron derived from pepsin-degraded food. The *H. pylori* genome encodes 11 proteins predicted to be involved in iron transport and 2 proteins thought to function as iron storage proteins (Alm *et al.*, 1999, Berg *et al.*, 1997, Tomb *et al.*, 1997, Van Vliet *et al.*, 2001).

C. Copper

Copper is a cofactor for several proteins involved in electron transport, oxidases, and hydroxylases, but may also contribute to the formation of reactive oxygen species (Rensing and Grass, 2003). *H. pylori* expresses several proteins which either are involved in copper transport or may act as copper chaperones.

D. Cobalt

The trace metal cobalt is a cofactor of the arginase enzyme, which plays an important role in nitrogen metabolism of *H. pylori* (McGee *et al.,* 1999,Mendz *et al.,* 1998) but also in modulating the immune response to *H. pylori* (Gobert *et al.,* 2002 , Gobert *et al.,* 2001). It has been noted that *H. pylori* is exquisitely sensitive to cobalt in vitro (95), and it has been suggested that cobalt may be used in nonantibiotic therapy of *H. pylori* infections (Bruggraber *et al.,* 2004, Bytzer and O'Morain, 2005).

3.2.5 Cell envelope, outer membrane, and LPS

The overall composition of the cell envelope of *H. pylori* is similar to that of other gram-negative bacteria. It consists of an inner (cytoplasmic) membrane, periplasm with peptidoglycan, and an outer membrane. The outer membrane consists of phospholipids and LPS. The *H. pylori* outer membrane phospholipid moiety contains cholesterol glucosides (Bukholm *et al.,* 1997, Haque *et al.,* 1996, Tannaes and Bukholm, 2005, Tannaes *et al.,* 2005), which is very rare in bacteria. LPS usually consists of lipid A, core oligosaccharide, and an O side chain. The lipid A moiety of *H. pylori* LPS has low biological activity compared to lipid A from other bacteria (Muotiala *et al.,* 1992).

The *H. pylori* genome encodes a large array of outer membrane proteins; adhesins, porins, iron transporters, flagellum associated proteins, and proteins of unknown function(Alm *et al.,* 2000, Doig *et al.,* 1995, Doig and Trust, 1994, Evans and Evans, 2000). The outer membrane of *H. pylori* often also contains urease and heat shock proteins, which are otherwise found only in the cytoplasm. Although specific export of these proteins cannot be completely excluded (Vanet and Labigne, 1998), this may be due to "altruistic lysis," a process wherein part of the population of bacterial cells lyses and releases its cytoplasmic proteins, which are subsequently used by surviving bacteria to coat their outer membrane with proteins from lysed cells (Marcus and Scott, 2001, Phadnis *et al.,* 1996).

3.3 Epidemiology

3.3.1 Prevalence and Geographical Distribution

The prevalence of *H. pylori* shows large geographical variations. In various developing countries, more than 80% of the population is *H. pylori* positive, even at young ages (Perez-Perez *et al.,* 2004). The prevalence of *H. pylori* in industrialized countries generally remains under 40% and is considerably lower in children and adolescents than in adults and elderly people (Pounder and Ng, 1995). Within geographical areas, the prevalence of *H. pylori* inversely correlates with socioeconomic status, in particular in relation to living conditions during childhood (Malaty and Graham, 1994). In Western countries, the prevalence of this bacterium is often considerably higher among first- and second-generation immigrants from the developing world (Perez-Perez *et al.,* 2005, Tsai *et al.,* 2005). While the prevalence of *H. pylori* infection in developing countries remains relatively constant, it is rapidly declining in the industrialized world (Genta, 2002). The latter is thought to be caused by the reduced chances of childhood infection due to improved hygiene and sanitation and the active elimination of carriership via antimicrobial treatment.

In developing countries, *H. pylori* infection rates rise rapidly in the first 5 years of life and remain constantly high thereafter, indicating that *H. pylori* is acquired early in childhood (Fiedorek *et al.,* 1991). However, in industrialized countries the prevalence of *H. pylori* infection is low early in childhood and slowly rises with increasing age. This increase results only to a small extent from *H. pylori* acquisition at later age. The incidence of new *H. pylori* infections among adults in the Western world is less than 0.5% per year; the higher prevalence of infection among the elderly thus reflects a birth cohort effect with higher infection rates in the past (Kuipers *et al.,* 1993, Parsonnet, 1995). The active elimination of *H. pylori* from the population and improved hygiene and housing conditions have resulted in a lower infection rate in children, which is reflected in the age distribution of this lifelong-colonizing bacterium (Kosunen *et al.,* 1997, Rehnberg-Laiho *et al.,* 2001, Roosendaal *et al.,* 1997). *H. pylori* is still a major issue in the developing countries, but the prevalence will eventually decline as it has already done in the developed countries (Tkachenko *et al.* 2007). Overall, new infection more commonly occurs in childhood and lasts for life unless specifically treated.

3.3.2 Transmission and Sources of Infection with Possible Risk Factors

The exact mechanisms whereby *H. pylori* is acquired are largely unknown. *H. pylori* has a narrow host range and is found almost exclusively in humans and some nonhuman primates.

H. pylori has on rare occasions been isolated from pet animals; thus, the presence of pets may be a risk factor for *H. pylori* infection (Brown *et al.,* 2001, Brown *et al.,* 2002, Dore *et al.,* 2001, Herbarth *et al.,* 2001). As conclusive evidence for zoonotic transmission of *H. pylori* is not yet available (Fox, 1995), new infections are thought to occur as a consequence of direct human-to-human transmission, via either an oral-oral or fecal-oral route or both and to some extent iatrogenic route in which infection spreads through tubes or endoscopes that is inserted in the gastric mucosa of one patient to another (Akamatsu *et al.,* 1996). Occupationally acquired infections transmitted from a patient to staff member have also been reported, especially among endoscopists and gastroenterologists (Liu *et al.,* 1996; Lin *et al.,* 1994)

H. pylori has been detected in saliva, vomitus, gastric refluxate, and feces (Allaker *et al.,* 2002, Ferguson *et al.,* 1999, Ferguson *et al.,* 1993, Kabir, 2004, Leung *et al.,* 1999, Parsonnet *et al.,* 1999, Sinha *et al.,* 2004), but there is no conclusive evidence for predominant transmission via any of these products. This may be due to the fact that most research on transmission has focused on adults.

It appeared that there was no clear increased risk for being a carrier of *H. pylori* among dentists, gastroenterologists, nurses, partners of an *H. pylori*-positive spouse, or visitors to a clinic for sexually transmitted diseases (Me´graud, 1995). As a result of these and other investigations, it is generally believed that acquisition mostly occurs in early childhood, most likely from close family members (Kivi *et al.,* 2003, Konno *et al.,* 2003, Raymond *et al.,* 2004, Rowland *et al.,* 2006, Taneike *et al.,* 2001, Van der Ende *et al.,* 1996). Premastication of food by the parent is an uncertain risk factor for transmission of *H. pylori* (Kurosawa *et al.,* 2000). Childhood crowding in and outside the family are all positively associated with *H. pylori* prevalence (Goodman and Correa 2000, Vincent *et al.,* 1994), whereas among adults crowding appears less important, with the exception of certain circumstances, such as among army recruits (Furesz *et al.,* 2004, Kyriazanos *et al.,* 2001, Rowland *et al.,* 2006).

Several studies have reported the presence of *H. pylori* DNA in environmental water sources (Enroth and Engstrand, 1995, Hegarty *et al.,* 1999, Queralt *et al.,* 2005), but this probably

reflects contamination with either naked DNA or dead *H. pylori* organisms. There is only a single report of *H. pylori* being successfully cultured from water, but this involved wastewater and as such may well represent fecal contamination of the water source (Lu *et al.,* 2002). Spread via fecal contaminants is supported by the occurrence of *H. pylori* infections among institutionalized young people during outbreaks of gastroenteritis (Laporte *et al.,* 2004). Other possible sources include contaminated food, as *H. pylori* may survive briefly on refrigerated food (Poms and Tatini, 2001). Coupled with the extreme sensitivity of *H. pylori* to atmospheric oxygen pressure, lack of nutrients, and temperatures outside the 34 to 40°C range (Kusters *et al.,* 1997), direct person-to-person transmission remains the most likely transmission route.

3.4 Pathogenesis

H. pylori induces chronic inflammation of the gastric mucosa, but only a proportion of infected individuals develop peptic ulcer disease or gastric carcinoma. Reasons underlying these observations include differences in bacterial pathogenicity as well as in host susceptibility. Numerous studies have provided new insight into *H. pylori* virulence factors, their interaction with the host and consequences in pathogenesis (Costa *et al.,* 2009).

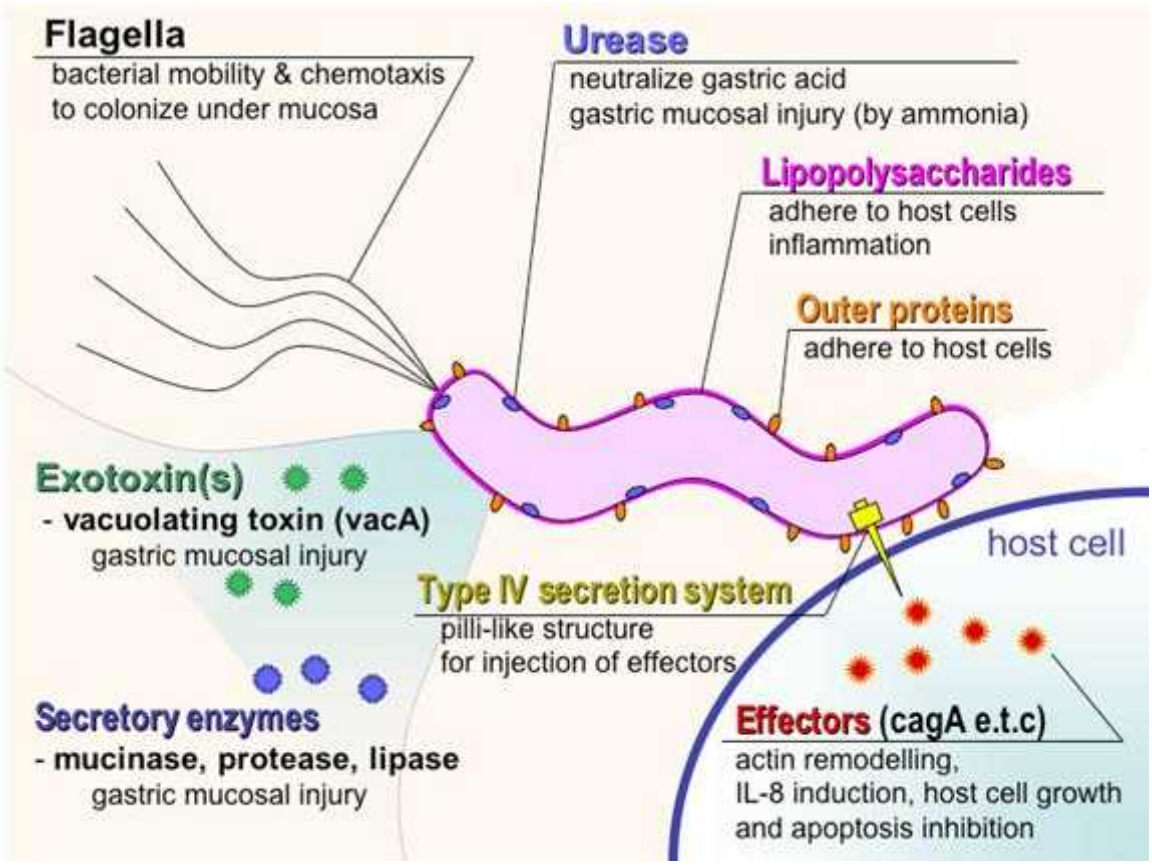
3.4.1 Pathogenecity and Virulence Factors

Investigators have identified several bacterial proteins necessary for colonization of the gastric mucosa by *H. pylori*, including proteins active in the transport of the organism to the surface of the mucosa (eg, flagellin, which is encoded on genes *flaA* and *flaB*) (Eaton *et al.,* 1996). Once in the presence of the gastric mucosa, bacteria induce a transient hypochlorhydria by an unknown mechanism. The urease enzyme produced by the bacteria alters the microenvironment of the organism to facilitate colonization (Rektorschek *et al.,* 1998). Adherence then occurs via interaction between cell-surface glycolipids and adhesins specific to *H. pylori* (Segal *et al.,* 1996). There also appears to be a role played by proteins called cecropins, which are produced by *H. pylori* and inhibit the growth of competing organisms (Putsep *et al.,* 1999), as well as by a P-type adenosinetriphosphatase, which helps prevent excessive alkalinization of the microenvironment by urease (Meichers *et al.,* 1995).

Once attached to gastric mucosa, *H. pylori* causes tissue injury by a complex cascade of events that depends on both the organism and the host. *H. pylori*, like all gram negative bacteria, has in its cell wall lipopolysaccharide, which acts to disrupt mucosal integrity

(Moran, 1996). Furthermore, *H. pylori* releases several pathogenic proteins that induce cell injury. For example, the CagA protein, produced by cytotoxic-associated gene A (*cagA*), is a highly immunogenic protein that may be associated with more severe clinical syndromes, such as duodenal ulcer and gastric adenocarcinoma (Censini *et al.*, 1995, Graham and Yamaoka, 2000). There is increasing evidence that CagA positivity is associated with an increased risk for distal, but not proximal, gastric adenocarcinoma (Wu *et al.*, 2001).

In addition, protein products of the vacuolating cytotoxin A gene (*vacA*) (Cover, 1996) and the A gene induced by contact with epithelium (*iceA*) are known to be associated with mucosal injury (Wu *et al.*, 1998).



(Source: Wikimedia Commons, the free media repository, 2007)

Figure 1: Schematic diagram of virulence factors of *Helicobacter pylori*.

Once colonization of the gastric mucosa has taken place, the immunogenic properties of *H. pylori* induce an inflammatory reaction with neutrophilic gastritis that ultimately results in the clinical manifestations of the infection. This process is mediated by host factors, including

interleukins 1, 2, 6, 8, and 12; interferon gamma; tumor necrosis factor–α; T and B lymphocytes; and phagocytic cells. These factors mediate injury through release of reactive oxygen species and inflammatory cytokines (Go and Crowe, 2000). *H. pylori* additionally appears to increase the rate of mucosal programmed cell death (also known as *apoptosis*) (Vorobjova *et al.,* 2001).

3.4.2 Effects on gastric physiology

In addition to producing local injury of gastric mucosa, *H. pylori* alters normal gastric secretion. Interestingly, the location and severity of the infection seem closely associated with the ultimate clinical outcome, most likely because of effects on gastric physiology. Many studies have shown that patients with a duodenal ulcer who are infected with *H. pylori* have an increased serum level of gastrin, which—in turn—leads to increased acid output (Peterson *et al.,* 1993). These patients tend to have a milder phenotypic expression of their gastritis, with inflammation mostly in the antrum or distal part of the stomach (Schultze *et al.,* 1998). In contrast, patients with gastric adenocarcinoma, a known complication of *H. pylori* infection, tend to have pangastritis, with involvement of the acid-secreting body of the stomach as well as the antrum. This condition leads to atrophy of parietal cells (which are responsible for producing acid) and gastrin-producing cells of the antrum (which stimulate acid secretion) and eventually produces achlorhydria. Patients with gastric adenocarcinoma also have impaired acid secretion in response to stimulation with gastrin (El-Omar *et al.,* 1997).

3.5 Clinical aspects of *H. pylori*-associated diseases

3.5.1 Anatomic features of stomach and duodenum: *H. pylori* perspective

H. pylori resides within the adherent mucus gel layer close to the epithelial surface. A continuous, adherent mucus gel layer with mucosal bicarbonate secretion and mucosal blood flow is the initial protective barrier in the stomach and duodenum against erosion by the gastric juice. The barrier function of the mucus layer in vivo depends on (i) its thickness, and (ii) its gel structure, a property which is linearly dependent on the polymeric mucin content (Allen *et al.,* 1997). Prostaglandins appear to be fundamental to the protection of the gastroduodenal mucosa (Rauws and Tytgat, 1989). Unfortunately neither sucralfate (Lambert *et al.,* 1985) nor prostaglandins (Ho *et al.,* 1986) have any effect on colonization in the stomach.

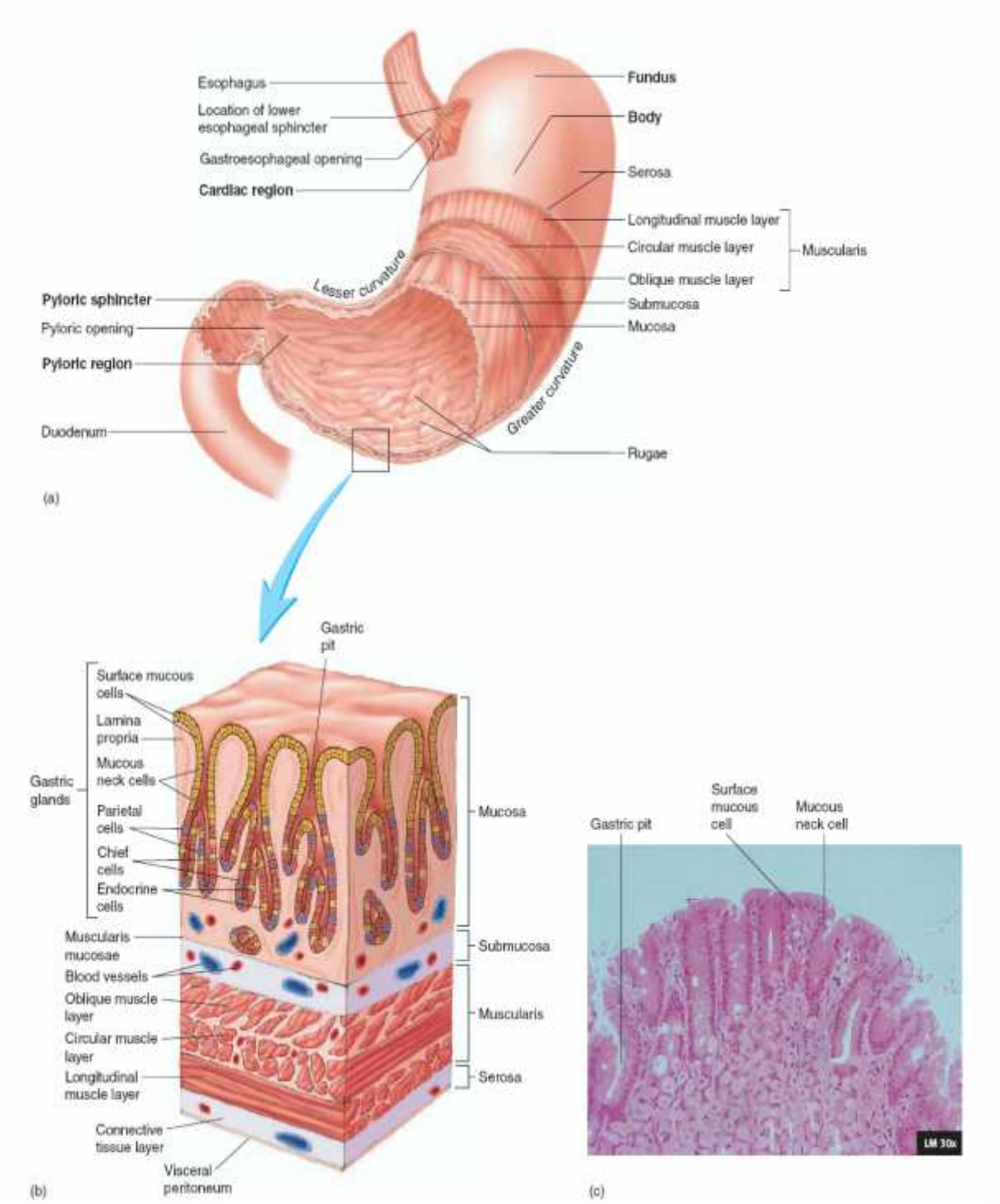


Figure 2: Anatomy and Histology of the Stomach (Seelay *et al.*, 2004)

(a) Cutway section reveals muscular layers and internal anatomy

(b) A section of the stomach wall that illustrates its histology, including several gastric pits and glands

(c) Photomicrograph of gastric glands

H. pylori infects only gastric type mucosal tissue but when such tissue occurs in the duodenum (gastric metaplasia), it can cause duodenitis, and acid and pepsin can then cause duodenal ulcer (Goodwin *et al.,* 1997). The most common site of a peptic ulcer is near the pylorus, usually on the duodenal side (i.e., a duodenal ulcer; 80% of peptic ulcers are duodenal). Ulcers occur less frequently along the lesser curvature of the stomach or at the point at which the esophagus enters the stomach (Seelay *et al.,* 2004).

In *H. pylori* infected patients there is an increase in fasting and in meal-stimulated serum gastrin levels (Graham and Qureshi, 2001).

3.5.2 Disease types

Although gastric colonization with *H. pylori* induces histologic gastritis in all infected individuals, only a minority develop any apparent clinical signs of this colonization. It is estimated that *H. pylori*-positive patients have a 10 to 20% lifetime risk of developing ulcer disease and a 1 to 2% risk of developing distal gastric cancer (Ernst and Gold, 2000, Kuipers, 1999, Kuipers *et al.,* 1995). The risk of development of these disorders in the presence of *H. pylori* infection depends on a variety of bacterial, host, and environmental factors that mostly relate to the pattern and severity of gastritis.

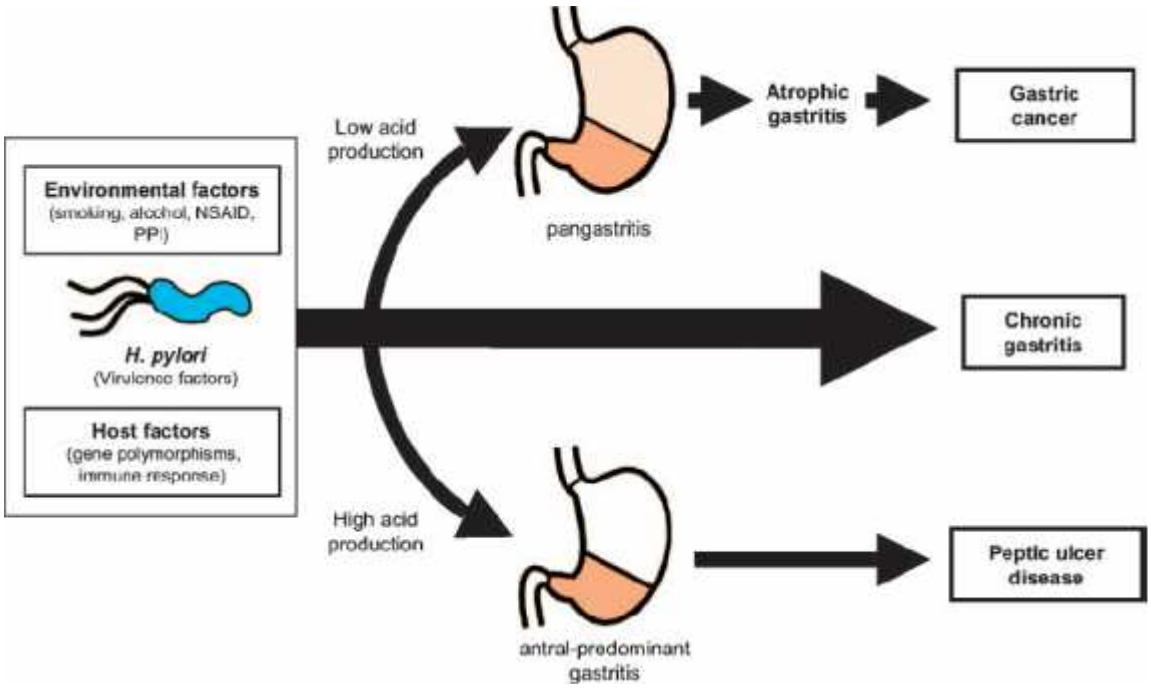


Figure 3: Schematic representation of the factors contributing to gastric pathology and disease outcome in *H. pylori* infection.

Colonization with *H. pylori* virtually always leads to infiltration of the gastric mucosa in both antrum and corpus with neutrophilic and mononuclear cells. This chronic active gastritis is the primary condition related to *H. pylori* colonization, and other *H. pylori*-associated disorders in particular result from this chronic inflammatory process.

I. Acute gastritis

Data on the acute phase of infection are scarce and largely come from reports of subjects who deliberately or inadvertently ingested *H. pylori* or underwent procedures with contaminated material (Graham *et al.,* 1988, Marshall *et al.,* 1985, Morris and Nicholson, 1987, Sobala *et al.,* 1991). The acute phase of colonization with *H. pylori* may be associated with transient nonspecific dyspeptic symptoms such as fullness, nausea, and vomiting, and with considerable inflammation of both the proximal and distal stomach mucosa, or pangastritis. This phase is often associated with hypochlorhydria, which can last for months.

II. Chronic gastritis

When colonization does become persistent, a close correlation exists between the level of acid secretion and the distribution of gastritis. This correlation results from the counteractive effects of acid on bacterial growth versus those of bacterial growth and associated mucosal inflammation on acid secretion and regulation. In subjects with intact acid secretion, *H. pylori* in particular colonizes the gastric antrum, where few acid-secretory parietal cells are present. This colonization pattern is associated with an antrum-predominant gastritis (Kuipers *et al.,* 1995). Although colonization with *H. pylori* is almost invariably associated with the presence of gastritis, and gastritis is mostly due to *H. pylori* colonization (Type B chronic gastritis), other causes of gastritis include infections such as cytomegalovirus, chronic idiopathic inflammatory and autoimmune disorders such as Crohn's disease and pernicious anemia, and chemical damage due to alcohol abuse or nonsteroidal anti-inflammatory drug (NSAID) use.

III. Peptic ulcer disease

Gastric or duodenal ulcers (commonly referred to as peptic ulcers) are defined as mucosal defects with a diameter of at least 0.5 cm penetrating through the muscularis mucosa. Gastric ulcers mostly occur along the lesser curvature of the stomach, in particular, at the transition from corpus to antrum mucosa (Veldhuyzen *et al.,* 1999). Duodenal ulcers usually occur in the duodenal bulb, which is the area most exposed to gastric acid. In Western countries, duodenal ulcers are approximately fourfold more common than gastric ulcers; elsewhere,

gastric ulcers are more common. Duodenal ulcers in particular occur between 20 and 50 years of age, while gastric ulcers predominantly arise in subjects over 40 years old.

a) Association with *H. pylori*

Both gastric and duodenal ulcer diseases are strongly related to *H. pylori*. In initial reports from all over the world in the first decade after the discovery of *H. pylori*, approximately 95% of duodenal ulcers and 85% of gastric ulcers occurred in the presence of *H. pylori* infection (Kuipers *et al.,* 1995). Approximately 50% of patients with *H. pylori*-associated peptic ulcer disease suffered ulcer recurrence within 1 year (Hentschell *et al.,* 1993, Rauws and Tytgat, 1990). Eradication of *H. pylori* dramatically changes the natural course of ulcer disease and almost completely prevents ulcer recurrence (Treiber and Lambert, 1998, Van der *et al.,* 1997).

b) *H. pylori* and NSAIDs

H. pylori and NSAIDs are the major causes of gastroduodenal ulcer disease. Their potential interaction in the induction of ulcer disease remains a controversial area. A thorough analysis of interaction data revealed that the ulcer-inducing effects of both risk factors are cumulative (Huang *et al.,* 2000). Eradication of *H. pylori* in chronic NSAID users decreases the incidence of ulcer disease.

IV. Non-ulcer dyspepsia

Nonulcer dyspepsia comprises a constellation of varied symptoms, including dysmotility-like, ulcer-like, and reflux-like symptoms. Many possible causes have been suggested for nonulcer dyspepsia, including lifestyle factors, stress, altered visceral sensation, increased serotonin sensitivity, alterations in gastric acid secretion and gastric emptying and *H. pylori* infection. A recent review also highlighted the role played by psychosocial impairment (eg, depression, somatization, anxiety) in patients with nonulcer dyspepsia (Olden and Drossman, 2000)

V. Atrophic gastritis, intestinal metaplasia, and gastric cancer

Chronic *H. pylori*-induced inflammation can eventually lead to loss of the normal gastric mucosal architecture, with destruction of gastric glands and replacement by fibrosis and intestinal- type epithelium. This process of atrophic gastritis and intestinal metaplasia occurs in approximately half of the *H. pylori*-colonized population, first in those subjects and at

those sites where inflammation is most severe (Kuipers *et al.,* 1995). The risk for atrophic gastritis depends on the distribution and pattern of chronic active inflammation. As such, subjects with decreased acid output show a more rapid progression towards atrophy (Kuipers *et al.,* 1996). Areas of gland loss and intestinal metaplasia extend with time multifocally, and although they do not give rise to any specific symptoms, they increase the risk for gastric cancer by 50 to 90 fold depending on the extent and severity of atrophy (Sipponen *et al.,* 1985).

Evidence that *H. pylori* increases the risk of gastric cancer development via the sequence of atrophy and metaplasia originates from various studies, in which it was shown that *H. pylori* positive subjects develop these conditions more often than do uninfected controls (Kuipers, 1998). This is supported by data that showed geographical associations between the prevalence of *H. pylori* and the incidence of gastric cancer (Forman *et al.,* 1990, The Eurogast Study Group, 1993). On the basis of these findings, it was estimated that *H. pylori* colonization increases the risk of gastric cancer approximately 10 fold and *H. pylori* was designated a class I carcinogen by the WHO (IARC, 1994).

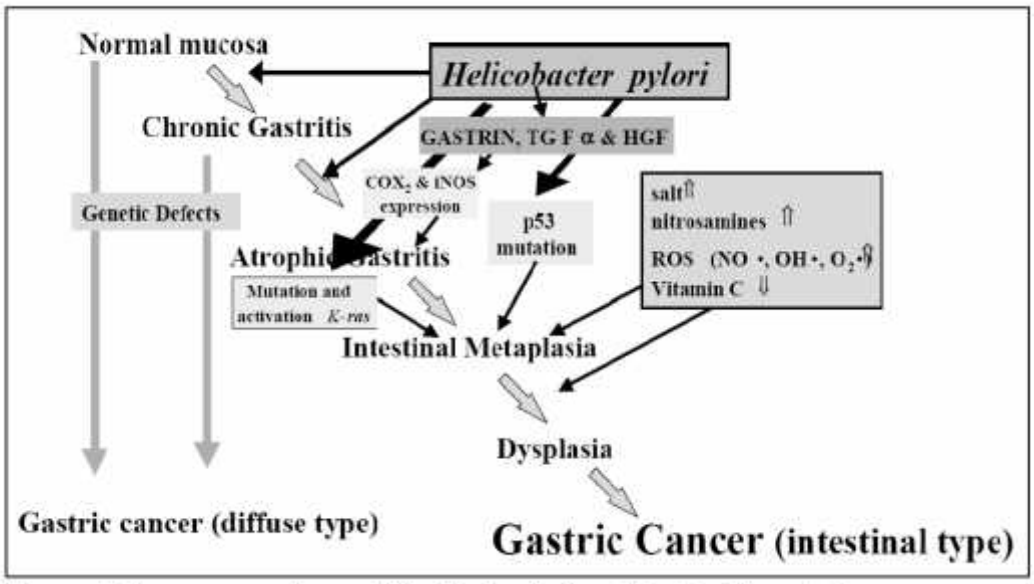


Figure 4: Correa cascade modified by including *H.pylori* in gastric cancerogenesis.

VI. Extragastroduodenal disorders

H. pylori has been linked to a variety of extragastric disorders. These include coronary heart disease, dermatological disorders such as rosacea and idiopathic urticaria, autoimmune thyroid disease and thrombocytopenic purpura, iron deficiency anemia, Raynaud's phenomenon, scleroderma, migraine, and Guillain-Barre´ syndrome. The underlying

hypothetical mechanisms include chronic low-grade activation of the coagulation cascade, accelerating atherosclerosis, and antigenic mimicry between *H. pylori* and host epitopes leading to autoimmune disorders (Gasbarrini *et al.*, 2004).

3.6 Diagnosis of *Helicobacter pylori* infection

Currently, there are several popular methods for detecting the presence of *H. pylori* infection, each having its own advantages, disadvantages, and limitations (Table 1).

Table 1: Diagnosis of *H. pylori* infection

Diagnostic method	Sensitivity and specificity[a]	Typical application	Remarks
Invasive methods			
Histology	> 95%	"Gold standard" in routine hospital diagnostics	Requires expert pathologist; also provides histological data on inflammation and atrophy
Culture biopsy	> 95%	Alternative gold standard	Allows for testing of antimicrobial sensitivity; requires specific microbiological expertise
Rapid urease test	> 90%	Cost-effective and rapid test	Requires an additional test for confirmation of *H.pylori* infection
Noninvasive methods			
Urea breath test	> 95%	Alternative gold standard	Very useful, reliable test to evaluate success of eradication treatment of *H. pylori*; limited availability due to requirement of expensive equipment
Faecal antigen test	> 90 %	Not widely used yet	Simple test but may not be reliable for evaluation of success of eradication treatment of *H. pylori*
Serology	80–90%	Mainly used for epidemiological studies	Insufficient reliability for routine screening; cannot prove ongoing infection due to immunological memory
[a] Global range, depending on regional variations and subjects.			

Source: Kusters *et al.,* 2006

Basically, the tests available for diagnosis can be separated according to whether or not endoscopic biopsy is necessary. Histologic evaluation, culture, polymerase chain reaction (PCR), and rapid urease tests are typically performed on tissue obtained at endoscopy. Alternatively, simple breath tests, serology, and stool assays are sometimes used, and trials investigating PCR amplification of saliva, feces, and dental plaque to detect the presence of *H. pylori* are ongoing (Bravos and Gilman, 2000).

3.6.1 Endoscopic aspects of *H. pylori* associated gastroduodenitis

Endoscopy is said to have limited reliability in detecting and scoring histological gastritis. Its main value is in obtaining specimens for histology after excluding oesophagitis, peptic ulcers, tumors etc. Confirmation of the endoscopic diagnosis of gastritis by histological inflammation has been reported in only 60 - 80 % of the cases. Although endoscopic features of *H. pylori* have been reported in the literature, there is still some debate over whether *H. pylori*-related gastritis can be diagnosed *via* endoscopic features alone. Most studies concluded that it is not possible to diagnose *H. pylori*-related gastritis on the basis of endoscopic findings (Bah *et al.,* 1995, Calabrese *et al.,* 1999, Loffeld *et al.,* 1999 and Redeen *et al.,* 2003)

3.6.2 Invasive or biopsy based methods

I. Histology

Histologic evaluation has traditionally been the gold standard method for diagnosing *H. pylori* infection. The disadvantage of this technique is the need for endoscopy to obtain tissue. Limitations also arise at times because of an inadequate number of biopsy specimens obtained or failure to obtain specimens from different areas of the stomach. In some cases, different staining techniques may be necessary, which can involve longer processing times and higher costs. However, histologic sampling does allow for definitive diagnosis of infection, as well as of the degree of inflammation or metaplasia and the presence/absence of MALT lymphoma or other gastric cancers in high-risk patients (El-Zimaity and Graham, 2002).

Biopsy specimens collected during endoscopy are immediately introduced into a fixative (10% formaldehyde) that maintains the morphology of the bacteria. Several stains such as Giemsa, Warthin Starry stain, Genta can be used. Immunostaining with a specific *H. pylori* antibody can also be used (Megraud and Lehours, 2007).

II. Culture

Because *H. pylori* is difficult to grow on culture media, the role of culture in diagnosis of the infection is limited mostly to research and epidemiologic considerations. Although costly, time-consuming, and labor intensive, culture does have a role in antibiotic susceptibility studies and studies of growth factors and metabolism (Perez-Perez, 2000).

The appropriate techniques for culturing *H. pylori* from endoscopic biopsies of the stomach and duodenum have been extensively reviewed. A variety of selective and non selective media are available commercially for this purpose.

III. Rapid Urease Test (RUT)

Rapid urease test takes advantage of the fact that *H. pylori* is a urease-producing organism (Mobley *et al.*, 1988). Samples obtained on endoscopy are placed in urea-containing medium; if urease is present, the urea will be broken down to carbon dioxide and ammonia, with a resultant increase in the pH of the medium and a subsequent color change in the pH-dependent indicator such as phenol red. This test has the advantages of being inexpensive, fast, and widely available. It is limited, however, by the possibility of false positive results; decreased urease activity, caused either by recent ingestion of antibiotic agents, bismuth compounds, proton pump inhibitors, or sucralfate or by bile reflux, can contribute to these false-positive results (Midolo *et al.*, 2000).

Various rapid urease tests are available commercially like CLO test, HP test and Pylori-Tek test. These provide comparable results with high sensitivity and specificity. However, simpler and cheaper in-house urease test medium giving similar results can be made.

IV. Molecular methods

With the advent of molecular tests, many exciting possibilities emerged for diagnosing and classifying *H. pylori* infection.

a) Polymerase Chain Reaction (PCR)

PCR allows identification of the organism in small samples with few bacteria present and entails no special requirements in processing and transport. Moreover, PCR can be performed rapidly and cost- effectively, and it can be used to identify different strains of bacteria for pathogenic and epidemiologic studies. As suggested earlier, PCR also is being evaluated for

its utility in identifying *H. pylori* in samples of dental plaque, saliva, and other easily sampled tissues (Bravos and Gilman, 2000).

The major limitation of PCR is that relatively few laboratories currently have the capability to run the assay. In addition, because PCR can detect segments of *H. pylori* DNA in the gastric mucosa of previously treated patients, false-positive results can occur, and errors in human interpretation of bands on electrophoretic gels can likewise lead to false-negative results.

b) Fluorescent in-situ hybridization (FISH)

FISH detects not only *H. pylori* infection but also resistance to antibiotics and presence of *cagA,* avoiding both culture and PCR. Fluorescent-labeled oligonucleotides bind to common *H. pylori* antigen, macrolide resistance antigen or *cagA*. After hybridisation the bacteria can be observed by fluorescence microscopy. The accuracy of the method has been shown to be high (Russmann *et al.,* 2001).

3.6.3 Non-invasive methods

I. Serology

In response to *H. pylori* infection, the immune system typically mounts a response through production of immunoglobulins to organism-specific antigens. These antibodies can be detected in serum or whole-blood samples easily obtained in a physician's office. The presence of IgG antibodies to *H. pylori* can be detected by use of a biochemical assay, and many different ones are commercially available nowadays. The sensitivity and specificity can be similar to that of the UBT, around 95% (Oksanen *et al.* 1998). In a meta-analysis of commercial kits, the medians of the sensitivity and specificity were 92% and 83% (Laheij *et al.* 1998). Serologic tests offer a fast, easy, and relatively inexpensive means of identifying patients who have been infected with the organism (Lerang *et al.,* 1998). Moreover serology assays the systemic response to entire stomach, therefore serological tests may be better in already established infection where the organism may not be detected bacteriologically.

Presently several rapid antibody tests (Immunochromatographic method) are in use in Nepal also which can have results within 5 – 10 minutes with comparable sensitivity and specificity to regular ELISAs. Hexagon *H. pylori,* is intended for the rapid, qualitative detection of *H. pylori* IgG, IgA and IgM antibodies in human serum, plasma and whole

blood as an aid in the specific diagnosis of *H. pylori* infections in patient with gastrointestinal symptoms. This kit is in practice in different hospitals of Nepal.

The test is based on the state of the art double antigen sandwich technology (3rd generation test). It employs *H. pylori* antigens which are fixed in the test line and also conjugated to colloidal gold in the mobile phase, and anti *H. pylori* antibodies (goat) in the control line. As the sample flows through the absorbent pad, human *H. pylori* antibodies are bound by the *H. pylori* dye conjugate to form an immunocomplex. This binds to the *H. pylori* antigens in the test line and produces a purple test line (T). Excess conjugate reacts in the control line (C) with the anti-human *H. pylori* antibodies (goat) forming a second purple line to demonstrate the correct function of the reagents.

Limitations

1. The tests are for use in the qualitative detection of *H. pylori* specific antibodies. They do not indicate the titre of the antibody in the specimen.
2. The test should be used to investigate patients with clinical signs and symptoms suggestive for *H. pylori* infections.
3. Specimens obtained too early during infection may not contain detectable levels of antibodies. If a negative result is obtained and infection is still suspected, a second sample should be obtained and tested 2-7 weeks later.
4. A positive result does not distinguish between acute infection and colonization by *H. pylori.* Positive results may occur even if the patient does not show typical symptoms.
5. Test results must always be evaluated in combination with other data and information available to the physician. If the test result is negative and *H. pylori* infection is suspected, other tests (i.e. histology, cell culture) should be performed as well.
6. All materials contaminated with patient specimens should be inactivated by validated procedures (autoclaving or chemical treatment) according to applicable regulations.

II. Urea Breath Test (UBT)

A urea breath test similarly relies on the urease activity of *H. pylori* to detect the presence of active infection. In this test, a patient with suspected infection ingests either 14C- labeled or 13C- labeled urea; 13C- labeled urea has the advantage of being nonradioactive and thus safer (theoretically) for children and women of childbearing age. Urease, if present, splits the urea into ammonia and isotope-labeled carbon dioxide; the carbon dioxide is absorbed

and eventually expired in the breath, where it is detected. The advantage of the method is its ability to assess the eradication of the bacterium after treatment and the possibility of using it in epidemiological studies especially in children. The drawbacks of the method are the high cost and false-negative results in case of treatment with proton pump inhibitors (Megraud and Lehours, 2007).

III. Stool antigen assay

H. pylori antigens can be detected in stools of *H. pylori* infected patients using ELISA based on monoclonal and polyclonal antibodies. The main advantage of this method is that it is applicable in large epidemiological studies in children.

IV. Urine antibody assay

It is simple and rapid and detects specific *H. pylori* IgG antibodies eliminated in urine. Both ELISA and immunoblotting have been used. Commercial tests have been developed in Japan: a standard ELISA, urinelisa and a rapid immunochromatographic test, Rapirun (Megraud and Lehours, 2007).

V.Salivary antibody assay

Detection of salivary *H. pylori* IgG antibodies is a simple diagnostic test. The best results are obtained when the serum titer is high as in duodenal ulcer patients or in children older than 5 years (Megraud and Lehours, 2007).

3.7 *Helicobacter pylori* eradication therapy

3.7.1 Challenges in the therapy

H. pylori lives in an acid niche, covered with mucus, where antibiotics cannot easily reach. Thus, even though *H. pylori* is sensitive to several antibiotics, only two antibiotics combined with a PPI or bismuth salt give satisfactory results. Resistance of *H. pylori* against the antibiotics used is the most important factor impairing the eradication results (Houben *et al.*, 1999). Compliance is another main factor, in which the eradication rate was reduced from 96% to 69% when 60% of the medication was taken (Malfertheiner, 1993; Wermeille *et al.*, 2002). As a consequence, the next regimen is faced with more resistant bacteria. Some studies have suggested that *H. pylori* is more easily eradicated in PUD than in NUD. However, studies with contrasting results have also been published, and a recent review of 22 studies could not confirm the difference between PUD and NUD (Huang *et al.*, 2005).

3.7.2 Indications for eradication therapy

In 1996 the European Helicobacter study group organized in Maastricht a meeting at which recommendations were given for diagnosis and therapy of *H. pylori*. This first meeting regarded only PUD and low-grade gastric MALT lymphoma as unequivocal indications for eradication. For gastritis with severe abnormalities and former gastric resection for gastric cancer, the evidence was only supportive, but therapy was still strongly recommended for these patients. Furthermore, eradication was advised in long term PPI treatment of gastro-oesophageal reflux disease, functional dyspepsia, family history of gastric cancer, NSAID therapy, post gastric surgery for PU and if the patient so wishes (Malfertheiner *et al.*, 1997). The second meeting, Maastricht 2-2000, further strongly recommended therapy in atrophic gastritis, for first-degree relatives of gastric cancer patients and if the patient so wishes (after a full consultation with their physician) (Malfertheiner *et al.,* 2002). The third consensus in 2007 recommended eradication therapy also in investigated NUD and uninvestigated dyspepsia (Malfertheiner *et al.*, 2007).

3.7.3 Impact of resistance

H. pylori diagnosis and susceptibility profile directs the applicability of the recommended treatment regimens. Hence the increasing prevalence of resistant strains makes culture and antibiotic susceptibility testing valuable to determine alternative treatment regimens after failure of the initial eradication regimens. Realizing the increasing prevalence of antimicrobial resistance around the globe and its potential negative impact on the efficacy of many treatment eradication regimens, it is important in clinical practice to determine the prevailing local antibiotic susceptibility patterns when choosing appropriate eradication regimens for *H. pylori* infections in empiric.

3.7.4 Antibiotic resistance

The antimicrobial resistance of *H. pylori* varies throughout the world. The antibiotics most often used in eradication therapy are amoxycillin, metronidazole, tetracycline, clarithromycin and recently levofloxacin. Resistance is the most important factor impairing eradication therapy.

I. Metronidazole

Metronidazole is metabolized by oxygen-sensitive nitroreductase enzymes of anaerobic bacteria into toxic metronidazole radicals that react with proteins, DNA and RNA, resulting in the death of the bacteria. Mutations in these genes may block the effect of nitroimidazoles. In *H. pylori* the main enzyme is oxygen-insensitive nicotineamide adenine dinucleotide phosphate (NADPH) nitroreductase. The toxic nitrosoderivatives this enzyme produces are not reoxidized by existing molecular oxygen, and cell damage ensues. Metronidazole resistance is mostly associated with mutations in the rdxA gene that encodes oxygen-insensitive NADPH nitroreductase. Mutations in the frxA gene, which encodes NAD(P)H-flavin oxidoreductase enhances the effect (Van der Wouden *et al.,* 2000, Bereswill *et al.,* 2003). Furthermore, mutations in the rdxA or frxA genes alone do not explain the resistance (Chisholm and Owen 2003, Marais *et al.,* 2003). As a result, metronidazole-based therapies can eradicate metronidazole-resistant strains, although not as effectively as sensitive strains (Lind *et al.,* 1999). Resistance to metronidazole in Europe and the USA is between 20% and 40%, while in the developing countries up to 80% (Glupczynski, 1998, Glupczynski *et al.,* 2001, Osato *et al.,* 2001). Women more often harbour resistant strains than men (Glupczynski *et al.,* 2001, Bruce *et al.,* 2006).

II. Clarithromycin

Resistance to macrolides is in most cases caused by one of three single-point mutations in the peptidyltransferase region of the 23S rRNA gene, mostly mutation A2143G or A2142G, when the adenine residue is replaced by guanine and less commonly by a cytosine residue (A2142C) (Oleastro *et al.,* 2003). These mutations decrease macrolide binding to the ribosomes. In a large European survey, resistance to clarithromycin was 9.9%. In the USA, about 10% of helicobacters are clarithromycin-resistant (Osato *et al.,* 2001, Meyer *et al.,* 2002). Resistance can be seen as a direct consequence of increased macrolide consumption (McMahon *et al.,* 2003). Clarithromycin resistance emerged in Estonia after clarithromycin became available, while the resistance frequencies in Japan correlated with macrolide sales (Loivukene *et al.,* 2002, Perez *et al.,* 2002). Thus, it is clear that clarithromycin resistance has increased and will continue to do so (Chisholm *et al.,* 2007; De Francesco *et al.,* 2007).

III. Amoxycillin

Fewer than 1% of helicobacters are resistant to amoxycillin (Megraud 2004). The mechanism is based on mutations in the genes coding for penicillin-binding protein and in consequence,

decreased affinity for amoxycillin leads to decreased accumulation of the antibiotic (Co and Schiller, 2006, Gerrits *et al.,* 2006). Still, in a Japanese study *H. pylori* strains resistant to amoxycillin appeared after the year 1996, while insensitive strains also increased, the importance of this phenomenon remains to be seen (Watanabe *et al.*, 2005).

IV. Tetracycline

Resistance to tetracycline is also very low, less than 1% (Wolle *et al.* 2002; Megraud 2004). A triple mutation AGA 926-928→TTC in the 16S rRNA gene is responsible. Since one or two mutations are not capable of producing resistance, tetracycline resistance is very rare despite its wide use (Gerrits *et al.* 2003).

V. Levofloxacin

Of the fluoroquinolones, levofloxacin has been tested in *H. pylori* eradication regimens. Resistance to fluoroquinolones is based on point mutations in the quinolone resistance-determining regions of *gyr*A.

3.7.5 In vitro antimicrobial susceptibility testing for *H.pylori*

Antimicrobial susceptibility testing methods for *H.pylori* is still under development. While a standardized methodology has been approved by the CLSI in the U.S., European investigators have choosen to pursue their own standard method that will only add to the already muddled waters of antimicrobial susceptibility testing. The Epsilometer (Etest), Kirby-Bauer disk diffusion, agar dilution and broth microdilution tests have been hailed as equivalent for assessing the antibiogram of *H. pylori* isolates (Von Recklinghausen and Ansorg, 1995, Hachem *et al.,* 1996, Piccolomini *et al.,* 1997, Weiss *et al.,* 1998 and Boyanova, 1999). Each test modality has its own advantages and disadvantages and their success is dependent both on the skill of the laboratory technician and the availability of the test components (Table 2).

Table 2: Test methods used for determining the antibiogram of *Helicobacter pylori*

Test method	Advantages	Disadvantages
Disk diffusion	Easy to perform Requires no special equipment Single isolates can be tested Inexpensive Widely available Can detect emergent resistant clone	No standard disk concentrations to use No standard effective diameters of inhibition Only one test isolate per plate Only qualitative susceptibility data (sensitive or resistant)
Broth microdilution	Multiple isolates can be tested/plate Endpoints easy to determine MBC determinations possible Quantitative endpoints (MIC)	Not widely available Not all antibiotics and/or concentrations available Custom plates are expensive No breakpoints for metronidazole, amoxicillin or tetracycline
Etest	Easy to perform Single isolates can be tested Quantitative endpoints (MIC) Can detect emergent resistant clone	Not all Etest strips FDA approved Testing of multiple isolates requires multiple plates Over estimates metronidazoe susceptibility High internal variability No breakpoints for metronidazole, amoxicillin or tetracycline
Agar dilution	Multiple concentrations can be tested CLSI standard methodology Quantitative endpoints (MIC)	Technically difficult to perform Expensive Each antibiotic requires multiple plates No breakpoints for metronidazole, amoxicillin or tetracycline
PCR	Multiple isolates can be tested at a time Rapid turnaround	Technically difficult to perform Expensive Qualitative endpoints Research tool, not generally available Requires specialized equipments

CHAPTER – IV

4. MATERIALS AND METHODS

4.1 Materials

Materials required for this study are cited in Appendix-VIII.

4.2 Methods

This cross sectional study, conducted from May to October, 2009, was carried out at c Health Research Laboratory, TUTH, Kathmandu and Norvic International Hospital and Medical College, Kathmandu.

Study group

Study group consists of 100 patients attending endoscopy unit of TUTH for the evaluation of upper gastro-intestinal symptoms.

Exclusion criteria

1) Presence of clinically significant associated conditions (hepatic, cardiorespiratory or renal diseases, pregnancy, insulin-dependent diabetes mellitus, neoplastic diseases or coagulopathy).
2) Patients on NSAIDs, bismuth compounds and antibiotics.
3) Previous gastric surgery.
4) Allergy to any of the drugs used in the study.

Ethic consideration

Approval for the study was obtained from the Institutional Review Board of the Institute of Medicine, Tribhuvan University.

An informed consent was taken from each patient for biopsy as well as blood sample.

A clinical profile of the patients including age, sex, upper gastrointestinal symptoms, underlying illness, history of alcohol consumption, smoking and medication was recorded before collecting the sample.

4.2.1 Endoscopic procedure

Endoscopy was executed by expert gastroenterologists using olympus video endoscope. The patient asked to come empty stomach for the procedure and was asked to lie on the left lateral position on the endoscopy table with the chin tucked against the chest and dentures if any were removed. The bite guard was placed securely between the teeth and the distal end of the endoscope was passed through the bite guard till it entered the stomach and then further inserted into the duodenum. The gastro-duodenal mucosa was examined for the presence of any lesion or ulcer. The endoscopic findings were noted as normal, gastritis (acute or mild and chronic) and peptic ulcer (gastric and duodenal ulcer).

4.2.2 Collection of sample

Biopsy forcep was inserted through the channel in the endoscope. Antral biopsy specimens were collected in case of normal and gastritis cases. In case of ulcer, biopsy specimens were collected from the adjacent mucosa. The 1-2 ml venous blood was drawn after biopsy samples for serology.

4.2.3 Processing of the sample

Three antral biopsies were taken in which first was directly inoculated into the in-house RUT tubes. The second was placed into a sterile container containing 10 % formalin for histology. The third one was placed into a sterile eppendorff tube containing 0.5 ml of normal saline to maintain humidity. In the laboratory, specimens were vortexed for the formation of a homogenate. Then it was cultured as well as gram-staining was performed. The collected blood was centrifuged at 3000 rpm for 10 minutes and serum was separated.

4.2.4. Rapid Urease Test

The in-house RUT was prepared by the use of 0.5 ml of a 10% unbuffered urea solution with 2 drops of a 1% phenol red solution in capped eppendroff tubes. The RUT tubes have been prepared an hour before the endoscopy.

The result was recorded within every 10 minutes, 1 hour and after 24 hours. The test was recorded as RUT positive when the colour of the solution changed from straw coloured to pink within 1 hour. If the test media turned pink after 24 hours, it was recorded as urease positive after 24 hours.

4.2.5 Culture

The homogenized specimen was heavily inoculated into non selective chocolate agar and selective columbia blood agar. The inoculated agar plates were put inside a dessicator where microaerophilic environment was created by conventional method i.e. Candle Jar with a pad of cotton soaked in water placed at the bottom and incubated at 37°C. The plates were examined for the bacterial growth after 2 days, 4 days and 10 days of incubation. If no growth was observed the plates were discarded after 10 days.

Bacterial isolates were identified by standard microbiological techniques that included colony morphology, gram staining and biochemical tests (catalase, oxidase and urease tests). The colonies of *H. pylori* were subcultured on 7% sheep blood agar plates until antibiotic susceptibility test was performed.

4.2.6 Antibiotic susceptibility test

The bacterial isolates were tested against amoxycillin, clarithromycin, metronidazole, tetracycline and levofloxacin by disk diffusion method (Modified Kirby-Bauer Method).

Inoculum preparation

A three day subculture of *H. pylori* was taken. Colonies were removed with a sterile cotton swab moistened in sterile nutrient broth and emulsified in a small volume of nutrient broth. The turbidity of this bacterial suspension was then matched with that of McFarland opacity standard of 3.

Inoculation and incubation

Thus standardized bacterial suspensions were inoculated onto the entire surface of the mueller hinton agar plates supplemented with 5% sheep blood with a swab and then it was allowed to dry. Antibiotic discs (amoxycillin, clarithromycin, levofloxacin, metronidazole and tetracycline) were placed aseptically onto the surface. All the plates were then incubated at 37°C under microaerophilic condition for 3 days.

Reading and interpretation of results

The diameter of inhibition zone was noted and the results were interpreted as sensitive and resistant as instructions provided by the manufacturer.

Table 3: Zone size interpretative chart for antibiotics against *H. pylori*

Antibiotics	Symbol	Disc content (mcg)	Diameter of zone of inhibition in mm	
			Sensitive (S)	Resistant (R)
Amoxycillin	AM	10	>16	< 16
Clarithromycin	CW	15	>21	< 21
Levofloxacin	LE	5	>19	< 19
Metronidazole	MT	5	>16	< 16
Tetracycline	T	30	>19	< 19

4.2.7 Histopathology

Histopathology was conducted in Norvic International Hospital and Medical College, Kathmandu.

Antral biopsy sample fixed in 10% formalin was processed overnight in a tissue processor to make paraffin blocks. Several 3-5 µm sections were cut with a microtome, followed by dewaxing the sections which were submerged in water for a while and finally staining with the modified Giemsa stain. Then the sections were washed, blotted, air dried and mounted. The stained sections were observed under microscope (100 x) for the presence of *H. pylori*.

Presence of *H. pylori* was distinctly visible as rod or comma shaped though little contrast occurred between the organism and tissue.

4.2.8 Serology

Serological status of *H. pylori* infection was tested with commercial *H. pylori* serology kits (HEXAGON H.PYLORI, Germany) following instructions of the manufacturer. The kit was used for the detection of *H. pylori* IgG, IgA and IgM antibodies in serum. It could detect anti *H. pylori* antibodies with a high sensitivity (95.9%) and specificity (89.6%) and a total agreement of 93.4% when compared with commercial *H pylori* IgG ELISA kit. 10 µl of the separated serum sample was added into the sample window **(S)** at the lower end of the test-kit. Then 3 full drops of diluent was added to the sample window, avoiding bubbles. Then the results were read within 10 minutes at a well lit place.

Interpretation of results

Negative

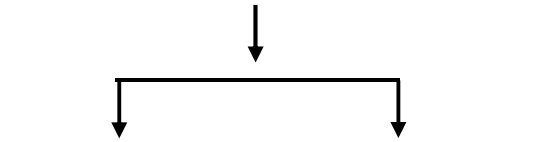
Only one purple control line **(C)** appeared in the upper part of the rectangular result window showing that the test was carried correctly.

Positive

A second purple test line **(T)** appearing in the lower part of the rectangular result window, indicated a positive result for *H.pylori* antibodies in the sample.

Even a weak T-line indicated a positive result.

Testing algorithm



Non-invasive test

(Serology)

Collection of 1-2 ml blood

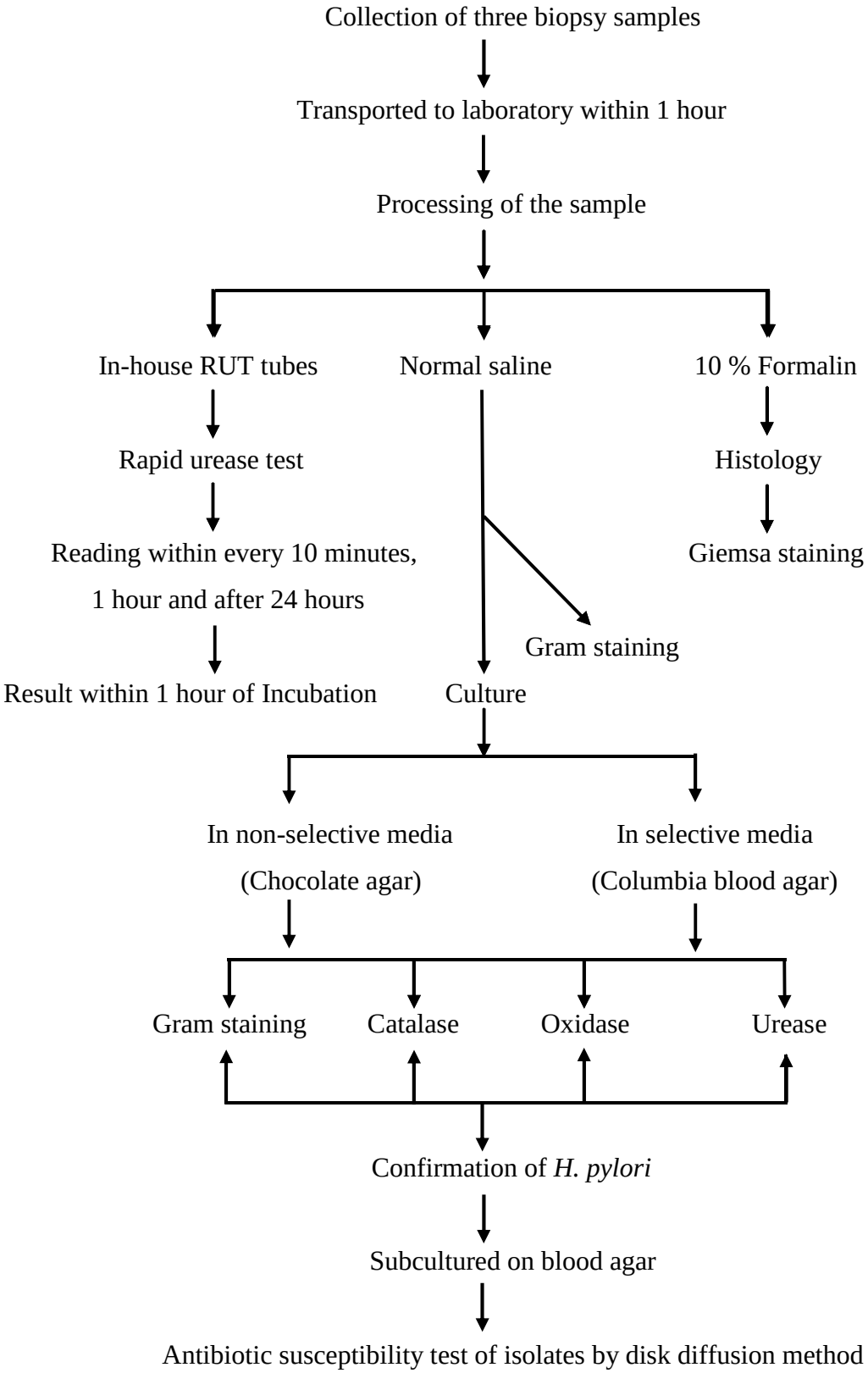
10 µl serum into sample window

3 full drops of diluent

Reading the result within 10 min after starting the test

Invasive tests

(Biopsy based tests)



CHAPTER - V

5. RESULTS

H. pylori positivity status was reflected as 32%, 32%, 47% and 63% by RUT, culture, histology and serology methods respectively among the 100 patients of this study.

5.1 Gender-wise distribution of *H. pylori* infection

The male to female ratio for *H. pylori* infection was 1.22:1.

Table 4: Gender-wise distribution of *H. pylori* positive subjects

Sex	Number of patients	Prevalence of *H. pylori*			
		RUT (%)	Culture (%)	Histology (%)	Serology (%)
Male	55	17 (30.9)	17 (30.9)	28 (50.9)	35 (63.6)
Female	45	15 (33.3)	15 (33.3)	19 (42.2)	28 (62.2)
Total	100	32	32	47	63

5.2 Age-wise distribution of *H. pylori* infection

The age of the subjects ranged from 11 to 77 years. The mean age of the patients was 38.9. The prevalence of *H. pylori* infection was highest in the age group of 20-29 years.

Table 5: Age-wise distribution of *H. pylori* positive subjects

Age group (years)	Number of patients	Prevalence of *H. pylori*			
		RUT (%)	Culture (%)	Histology (%)	Serology (%)
11-20	10	5 (50)	2 (20)	6 (60)	7 (70)
20-29	22	10 (45.4)	11 (50)	12 (54.5)	18 (81.8)
29-38	20	3 (15)	7 (35)	10 (50)	13 (65)
38-47	20	9 (45)	6 (30)	9 (45)	11 (17.4)
47-56	14	3 (21.4)	3 (21.4)	7 (50)	7 (50)
56-65	6	1 (16.6)	2 (33.3)	2 (33.3)	4 (66.6)
65-74	4	1 (25)	1 (25)	1 (25)	1 (25)
74-83	4	-	-	-	2 (50)
Total	100	32	32	47	63

5.3 Association of *H. pylori* infection with endoscopic findings

The overall prevalence of *H. pylori* infection was higher in duodenal ulcer cases whereas the normal cases from endoscopic findings also demonstrated remarkable *H. pylori* infection.

Table 6: Association of *H. pylori* infection with endoscopic findings

Endoscopic findings	Number of patients	Prevalence of *H. pylori*			
		RUT (%)	Culture (%)	Histology (%)	Serology (%)
Antral gastritis	43	8 (18.6)	11 (25.5)	17 (39.5)	24 (55.8)
Duodenal ulcer	7	5 (71.4)	5 (71.4)	5 (71.4)	6 (85.7)
Erosive gastritis	11	5 (45.4)	6 (54.5)	7 (63.6)	8 (72.7)
Erosive pangastritis	2	1 (50)	1 (50)	1 (50)	2 (100)
Fundal gastritis	4	3 (75)	1 (25)	2 (50)	3 (75)
Gastric ulcer	6	1 (16.6)	2 (33.3)	1 (16.6)	3 (50)
Gastroduodenitis	5	1 (20)	2 (40)	2 (40)	2 (40)
Normal	22	8 (36.3)	4 (18.1)	12 (54.5)	15 (68.1)
Total	100	32	32	47	63

5.4 Prevalence of *H. pylori* infection

For the definition of a positive *H. pylori* status, the results of a RUT, histological examination and microbiological cultures were used. A patient was considered to be positive with respect to *H. pylori* infection when at least two of these three tests gave positive results (Weingart *et al.*, 2004).

Table 7: Prevalence of *H. pylori* infection

Endoscopic findings	**Number of patients**	***H. pylori* positive (%)**
Antral gastritis	43	11 (25.5)
Duodenal ulcer	7	5 (71.4)
Erosive gastritis	11	6 (54.5)
Erosive pangastritis	2	1 (50)
Fundal gastritis	4	2 (50)
Gastric ulcer	6	1 (16.6)
Gastroduodenal gastritis	5	2 (40)
Normal	22	8 (36.3)
Total	100	36

Hence the prevalence of *H. pylori* infection was found to be 36% with duodenal ulcer holding the top position.

5.5 Seroprevalence of *H. pylori* infection

The seroprevalence of *H. pylori* was 63%. Then efficacy of the serological test with other three tests i.e. RUT, culture and histology were compared by applying chi-square test in which result was found to be significantly associated.

5.6 Result of antibiotic susceptibility test

Only 30 from 32 culture positive cases were efficiently subcultured and further processed for antibiotic susceptibility testing.

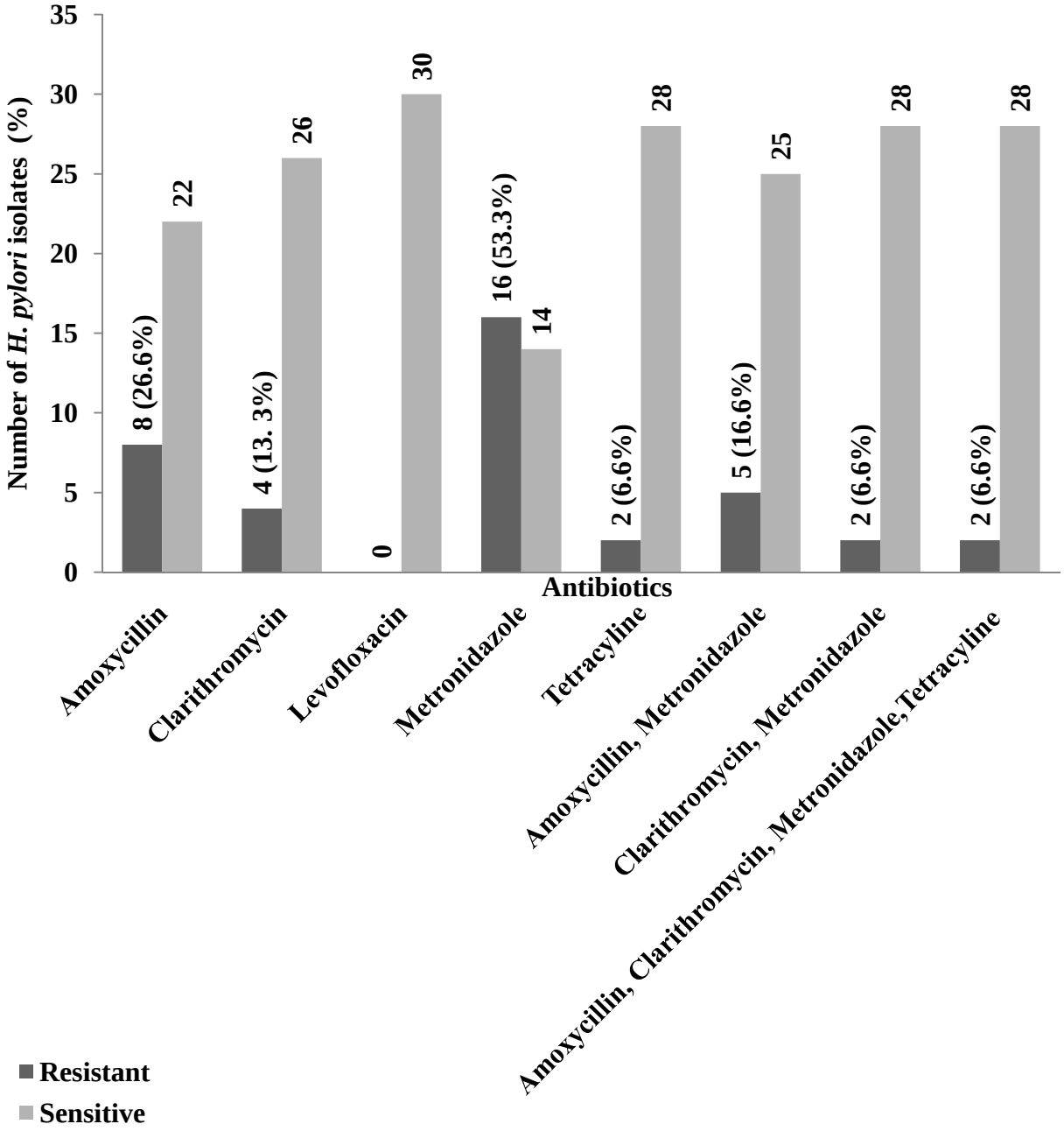


Figure 5: Antibiotic susceptibility testing of *H. pylori* isolates.

Resistance to metronidazole was found to be the highest securing 53.3%, followed by amoxycillin (26.6%), clarithromycin (13.3%) and tetracycline (6.6%) while all isolates were sensitive to levofloxacin. Multi drug resistance was also observed in which amoxycillin and metronidazole resistance was 16.6% and clarithromycin and metronidazole 6.6%. 2 strains were resistant to four drugs namely amoxycillin, clarithromycin, metronidazole and tetracycline.

CHAPTER- VI

6. DISCUSSION AND CONCLUSION

6.1 Discussion

With the goal of assessing the prevalence of *H.pylori* infection regarding gastrointestinal diseases in Nepal and antibiotic susceptibility of the isolates to contemporary antibiotics, the current study was completed proficiently by employing the four diagnostic tests: rapid urease test, culture, histology and serology.

This study divulged the prevalence of *H.pylori* infection as 36% with the consideration that at least two of the three already established tests (RUT, culture and histology) positive. However seroprevalence was obtained to be 63%. Numerous studies have applied different diagnostic tests to evaluate the prevalence of *H.pylori* infection. Certain studies carried out in Nepal presented the prevalence of *H. pylori* infection as 56.8% (Kawasaki *et al.,* 1998) using serology, 39.2% (Subedi, 2001) using histopathology and/or culture, 33.9% (Makaju *et al.,* 2005) using histopathology, 29.5% (Rai *et al.,* 2006) using RUT, culture and PCR, 25.5% (Adhikari, 2008) using RUT and/or culture.

The prevalence of 75.4% of *H. pylori* using PCR has been reported in Sri Lanka (Fernando *et al.,* 2002*).* A high prevalence of *H. pylori* has also been observed in India (88%) (Graham *et al.,* 2009). An overall prevalence of 84.6% using PCR is observed in Pakistan. In Bangladesh 90% are positive for *H. pylori,* both as assessed by a highly specific and sensitive stool antigen test and by serology (Bhuiyan, 2010). Very recently, the prevalence of *H. pylori* is also reported to be relatively high (50%) in China (Zhang *et al.,* 2009). In Africa (e.g. Ethiopia, Gambia and Nigeria), >90% of the populations are infected with *H. pylori* (Holcombe *et al.,* 1993, Lindkvist *et al.,* 1996, Thomas *et al.,* 1999). In Latin America, e.g. Chile and Mexico, around 70% are shown to be infected with *H. pylori* (O'Rourke *et al.,* 2003). On the other hand, in industrialized countries like Sweden, only 11% has been reported to be infected with *H. pylori* (Thjodleifsson *et al.,* 2007). Slightly higher prevalence has been observed in other Western countries such as Italy (43%) and the US (20%) (Rupnow *et al.,* 2000). From these data it is clear that more than 50 percent of the population

worldwide is infected with *H. pylori* with a higher prevalence in developing countries and in groups with poor socio-economic and hygienic status than in developed areas.

The lower prevalence (36%) observed in this particular study as compared to other developing countries can be explained by different social and cultural practices exist in Nepal. By comparing the present study with Subedi, 2001 as both studies have comparable tests parameters; the picture of prevalence shows the decline from 39.2% to 36% by 3.2%. However seroprevalence observed in the study shows an increase in prevalence from 56.8% (Kawasaki *et al.,* 1998) to 63% by 6.2%. Hence seroprevalence study reported higher prevalence of *H. pylori* infection as compared to biopsy-based studies in Nepal as serology not only reflects active infection but also past infection. The prevalence of *H. pylori* infection varies between regions of the same country or other parts of the world. Many variations, including studied populations, bacterial strains, geographic locations, the efficacy of diagnostic methods, environmental, and socio-economic factors may contribute which make it difficult to interpret existing data (Nawapon *et al.,* 2005).

The male to female ratio for *H. pylori* infection was 1.22:1. There was no gender bias in prevalence of the bacterium as earlier studies (Kawasaki *et al.,* 1998, Subedi, 2001, Nawapon *et al.,* 2005, Adhikari, 2008, Mishra *et al.,* 2008).

The prevalence of *H. pylori* infection was the highest (54.5%) in the age groups of 20-29 years including the seroprevalence. Subedi (2001) reported higher prevalence in younger age group. Rai *et al.,* (2006) reported higher prevalence in 36-50 years. Adhikari (2008) observed higher prevalence in the age group of 26-35 years. In developing countries, the prevalence of *H. pylori* infection is high in the 20-30 year age group and the prevalence of the infection varies between subpopulations within the same country, especially in relation to age (Malaty, 2007). Hence the study matches the prevalence scenario of developing countries.

H. pylori infection was higher in duodenal ulcer cases (71.4%) that are in consistent with the study of Subedi (2001), Adhikari (2008) as well as many other studies. 36.3% *H. pylori* was positive in endoscopically normal stomach which was 10.3% in Subedi (2001) and 27.3% in Adhikari (2008) while 65.6% in Khan *et al.,* (1999), 68% in Hassan and Zaigham (2007), 72.2% in Jemilohun *et al.,* (2010). These data confer that there is a great deal of ignorance about the normal macroscopic appearance of the gastric mucosal lining. In all probability

many of the appearance which endoscopists interpret as "Normal" are presumably not normal (Fung *et al.,* 1979, Taor *et al.,* 1975). Endoscopic findings such as erythema are frequently labelled as gastritis despite a long recognized lack of evidence supporting a correlation between endoscopic features and histologic gastritis (Sauerbruch *et al.,* 1984). It's still controversial whether certain macroscopic endoscopic features can be used to diagnose *H. pylori* related gastritis. The literature dealing with this subject is confusing, because of the lack of precise terminology, no large control trials, major discrepancies in interpretations of macroscopic changes and poor correlation of the macroscopic appearance and histological finding of gastritis (Khan *et al.,* 1999).

Invasive tests were the first to be used in *H. pylori* diagnosis. The inconvenience of the upper GI endoscopy needed to obtain the biopsy samples for the tests led to the development of noninvasive tests. Other means to obtain *H. pylori* samples from the stomach have been introduced (nasogastric tube, string test and gastric brush), but they have not displaced endoscopy.

A “home-made” unbuffered rapid urease test (RUT) is an accurate and inexpensive method for routine detection of *H. pylori* (Thillainayagam *et al.,* 1991). The rapid urease test (RUT) is based on the urease activity of *H. pylori*. Helicobacters in the gastric biopsy samples placed in the urea-containing media produce ammonia, raise the pH of the media and change the colour of the pH indicator (phenol red) from yellow at pH 6.8 to magenta at pH 8.4. The most rapid modern tests give positive results in minutes, enabling endoscopists to begin eradication therapy immediately after endoscopy (Goh *et al.,* 2007). The tests were considered as true negatives after incubation for 24 hours. Such two cases were confronted in this study too.

When interfering factors are excluded, the sensitivity and specificity are over 90% (Misra *et al.,* 1999). However, any condition reducing *H. pylori* density in the stomach reduces the sensitivity of the test. Use of PPIs, by increasing the gastric pH, makes the environment less suitable for *H. pylori* and reduces the density of the bacteria. The change is most striking in the antrum and helicobacters may be found only in the fundus. The habit of taking the biopsy for the RUT only in the antrum is inadequate in this situation. If the patient uses PPIs, the sensitivity of the RUT may even fall below 50% (Yakoob *et al.,* 2005). Bleeding from GUs or DUs or presence of blood in the stomach from any source is considered to decrease the sensitivity of the RUT to 60–70% (Gisbert and Abraira, 2006). Still, the RUT was as

sensitive as histology and culture in this situation. Use of PPIs before upper endoscopy may in part explain the inferiority of the results obtained with the RUT among these patients (Udd *et al,*. 2003). The longstanding infection can lead to atrophic change, especially in the gastric antrum, but also in the gastric body. Subsequently, an increase in gastric pH in the body atrophy reduces the density of *H. pylori* below the detection level of the RUT (Tucci *et al.,* 2005). On the other side, the nonacidic ventricle may harbour other bacteria with urease activity, giving false-positive results (Brandi *et al.,* 2006). Chronic renal failure, in which the prevalence of *H. pylori* infection is lower, may also decrease the sensitivity of the RUT (Misra *et al.,* 1999). After a failed eradication therapy, *H. pylori* may require over 4 weeks to recover to the level detected by the RUT (Laine *et al.,* 1998).

Histology was the first means of recognizing *H. pylori*, preceding the other tools by over 100 years. It is still considered the gold standard for diagnosing *H. pylori* infection. The updated Sydney system was created for evaluation of the biopsy samples (Dixon *et al.,* 1996). The value of histology is not only based on detecting *H. pylori*, but also on finding atrophic gastritis and premalignant and malignant changes in *H. pylori* gastritis, in addition to other diseases not associated with *H. pylori*. In spite of undetectable *H. pylori*, the presence of active gastritis indicates ongoing infection and suggests the need for further diagnostic tests. On the other hand, a normal histology practically excludes the infection (Megraud and Lehours, 2007).

The sensitivity of histology is compromised under the same conditions as with RUT. In atrophic gastritis the density of *H. pylori* can be too low to be detected. The use of PPIs transfers the infection to the upper part of the stomach and reduces the amount of the bacteria (Nakshabendi *et al.,* 1996). Antibiotic therapies can reduce the amount of *H. pylori*. Upper GI bleeding reduces the sensitivity (Gisbert and Abraira, 2006). *H. pylori* infection can be patchy and more biopsies may be needed for detection. Under these conditions, Giemsa and silver staining make it easier to detect *H. pylori* (Dixon *et al.,* 1996, Anim *et al.,* 2000, Rotimi *et al.,* 2000).

Culturing gastric biopsies is an almost 100% specific means to find *H. pylori* infection. However, the sensitivity is lower for several reasons, viz. sampling error due to patchy distribution of the organism in the gastric mucosa, contamination of the biopsy forceps, presence of oro-pharyngeal flora, transport condition, nature of media, atmospheric condition

of the culture and also on the experience and preference of the lab. Selective as well as non-selective media are commercially available for the isolation of *H. pylori*. In this study, both non-selective (chocolate agar) and selective media (Coloumbia Blood agar medium) were used. Selective medium contains antibiotics such as vancomycin, amphotericin B and trimethoprim which restrict the overgrowth of contaminating microorganisms. Cefsulodin specifically inhibits the Pseudomonads. Non-selective medium at the same time allows the growth of some *H. pylori* strains that may be suppressed by the antibiotic supplements (Ansorg *et al.,* 1991; Hua *et al.,* 1998).

Culturing a slowly growing microorganism under microaerophilic conditions is a demanding process. In optimal circumstances the sensitivity can be as much as 95% (Perez-Perez 2000, Matsukura *et al.*, 2004, Megraud and Lehours, 2007). RUT tests, which also serve as a transportation medium for culture, make it more convenient to obtain cultures at the first endoscopy. Culturing has the same problems in sensitivity as the RUT and histology. When the mucosal bacterial density is low, it may remain negative. In a bleeding PU the sensitivity was only 45% (Gisbert and Abraira, 2006). After antibiotic therapies, it is recommended to wait 1 month before culturing. It may require 8 weeks before culturing succeeds after failed eradication therapy (Laine *et al.,* 2000).

Coincidentally the prevalence of *H. pylori* by both RUT and culture was observed 32% and histoprevalence was 47% which is more or less in consistent with Subedi (2001) (39% RUT, 36% culture and 41% histology). This reflects RUT is as good as culture in diagnostic value. So RUT should be in practice along with histology for confirmation of *H. pylori* in the particular biopsy sample.

A major advantage of serology is that the former use of antibiotics or PPIs and upper GI bleeding do not affect the test, in contrast to invasive tests. In gastric body atrophy, serology is the most reliable means of discovering *H. pylori* infection (Kokkola *et al.,* 2000). When histology shows body atrophy, serology is recommended to exclude *H.pylori*. Serology can also be used in confirming the result of *H. pylori* eradication therapy. A decrease of at least 40% in the IgG antibody level 4 months after therapy from the pretreatment level is diagnostic of treatment success. Since in most cases IgG antibodies remain detectable for years after eradication, paired serum samples are always needed in this context (Kosunen *et al,*. 1992). This may be a prominent reason for higher seroprevalence rate including this study

with 63%. Serology also has some drawbacks. It is not recommended for use in populations where *H. pylori* prevalence is lower than 30%, due to false-positive results. Among young children, the accuracy is lower than among adults (Kolho *et al.,* 2002, Bonamico *et al.* 2004, Sherman, 2004).

H. pylori is the main cause of most gastroduodenal diseases which has been a major breakthrough in gastroenterology. It has dramatically changed management of these diseases which are now considered as infectious diseases and treated with antibiotics. An indiscriminate use of antibiotics to eradicate *H. pylori* also from healthy carriers would lead to severe problems with bacterial resistance against these important drugs. Therefore, treatment against *H. pylori* should be restrictively in patients without documented gastric or duodenal ulcer disease.

H. pylori is sensitive to several antibiotics, however, their accessibility into the *H. pylori* regime is not an easy task. That's why only two antibiotics combined with a PPI or bismuth salt give satisfactory results. Bacterial resistance and patient non-compliance are the two major causes of treatment failure and their overall effect on the delivery of medical care. Thus, pretreatment susceptibility testing not only enhances the probability of a successful treatment outcome but also reduces the costs of treatment because expenses related to evaluation of the patient and additional drugs would not be incurred. Antimicrobial resistance is not restricted to *H. pylori*, but extends to all other organisms that come into contact with these antibiotics. Therefore, while pretreatment susceptibility testing has a significant impact on treatment outcome, a secondary but equally important issue is the decreased possibility for emergence of more resistant strains by the use of appropriate therapy. The judicious use of the multiple drug therapies will further extend the useful life of these agents for treatment of not only *H. pylori* infection but other infections as well.

Realizing the increasing prevalence of antimicrobial resistance around the globe and its potential negative impact on the efficacy of many treatment eradication regimens, it is important in clinical practice to determine the prevailing local antibiotic susceptibility patterns when choosing appropriate eradication regimens for *H. pylori* infections in empiric and if the infecting strain is resistant, successful eradication is compromised. Metronidazole, clarithromycin, amoxycillin and tetracycline are regularly prescribed for the treatment of *H. pylori* infection in Nepal. Resistance to these antibiotics generally varies between

geographical regions and among subgroups within a population. Local prevalence of antimicrobial resistance determines the first line antibiotic to be included in a treatment regimen. In Nepal due to methodological difficulties in isolating the organism, detection of the organism by culture methods has not been eminent. So, data regarding antibiotic resistance must be collected from previous studies.

Being simple, inexpensive and routinely applicable technique, disk diffusion is the method of choice in this study for antibiotic susceptibility assessment. Five antibiotics namely amoxycillin (10 mcg), clarithromycin (15 mcg), levofloxacin (5 mcg), metronidazole (5 mcg) and tetracycilne (15 mcg) were used. The sensitivity of antibiotics was polarized to the very sensitive and very resistant ends, leaving little room to speculate about the grey zone between sensitivity and resistance i.e. intermediate. Of total 53.3% were found metronidazole resistant followed by amoxycillin (26.6%), clarithromycin (13.3%) and tetracycline (6.6%) while all isolates were sensitive to levofloxacin. Multi drug resistance was also observed in which amoxycillin and metronidazole resistance was 16.6% and clarithromycin and metronidazole 6.6%. Two strains were resistant to four drugs: amoxycillin, clarithromycin, metronidazole and tetracycline.

To our knowledge, levofloxacin is the first time being evaluated for the susceptibility of *H. pylori* as it retains its activity in vitro when *H. pylori* strains are resistant to clarithromycin and metronidazole. These favourable results have been confirmed in vivo, indicating that most of the patients with both metronidazole and clarithromycin resistant are cured with the levofloxacin containing regimens (Getta *et al*., 2005). Moreover levofloxacin containing second line therapies represent an encouraging strategy for eradication failure as some studies have demonstrated that levofloxacin has, in vitro, remarkable activity against *H. pylori* and that primary resistant to such antibiotic are infrequent (Bilandi *et al*., 2004).

The previous study by Thapa, 1996 reported that 6% of *H. pylori* isolates were resistant to amoxycillin, 4% to tetracycline and 30% to metronidazole. Similarly by Subedi (2001) showed that 50% of the isolates were resistant to metronidazole, 6.6% were resistant to amoxycillin and 3.3% were resistant to clarithromycin. 6.6% of the isolates were resistant to both amoxycillin and metronidazole whereas clarithromycin and metronidazole resistance was not observed. The study by Adhikari, 2008 reported that 53.8% of the isolates were metronidazole resistant, 15.3% were amoxycillin resistant while all were sensitive to

clarithromycin. Further, none of the isolates were multi-drug resistant. This reflects the resistance scenario of Nepal. Metronidazole resistance is prevalent since 2001, followed by amoxycillin, clarithromycin and tetracycline.

Resistance to metronidazole in Europe and the USA is between 20% and 40%, but wide use of metronidazole for parasitic infections increases resistance in the developing countries up to 80% (Glupczynski, 1998, Glupczynski *et al.,* 2001, Osato *et al.,* 2001). In developing countries, the prevalence of metronidazole resistance among *H. pylori* isolates was much higher, 50 to 80% (Nahar *et al.,* 2004, Torres *et al.,* 2001 and Wheeldon *et al.,* 2004). In Japan, exceptionally low resistance rates are seen (12.4%), again with local differences. Women more often harbour resistant strains than men, most probably due to therapies for gynaecologic infections (Glupczynski *et al.,* 2001, Bruce *et al.,* 2006). Different mechanisms of metronidazole resistance in *H. pylori* have been put forward. Mutations in *rdx A* gene and *frx A* gene as well as involvement of Tol C efflux pump appeared to play a role in resistance to this antibiotic.

Amoxycillin resistance was not considered important until recently identified in the United States, Canada and Italy (Dore *et al.,* 1997, Fedorak *et al.,* 1997). Amoxycillin is one of the most commonly used antimicrobial agents in Nepal in recent years for respiratory tract infections, urinary tract infections etc. Amoxycillin resistance develops due to structural alterations in one of the penicillin-binding proteins or changes in other proteins involved in cell wall synthesis.

The reported prevalence of resistance to clarithromycin ranges between 0-15% in most countries (Debets-Ossenkopp *et al.,* 1999, Toracchio and Marzio *et al.,* 2003). In Bangladesh, clarithromycin was introduced in the late 1990s, and it has been widely used for eradication of *H. pylori*. Previous use of macrolides has been shown to be associated with *H. pylori* resistance to clarithromycin.

Resistance to tetracycline is very low, less than 1% in western countries (Wolle *et al.,* 2002, Megraud 2004) whereas resistance ranges between 5 and 59% in Asian countries (Kwon *et al.,* 2000, Thyagarajan *et al.,* 2003, Wu *et al.,* 2000). A triple mutation in the 16S rRNA gene is responsible for the resistance.

Antibiotic resistance is undisputedly an emerging problem for the treatment of *H. pylori* infected patients in Nepal. The present study also demonstrates the need for continuous monitoring of the antimicrobial susceptibility in *H. pylori* that will certainly guide the clinicians for determination of optimal treatment regimens as multi drug resistant strains are already in the act. Hence more effective regimens are needed as *H. pylori* predisposes to various serious conditions like peptic ulcer and gastric cancer without any delay though the disease has a low mortality, it results into substantial human suffering and hence loss of manpower (Berry and Sagar, 2006).

6.2 Conclusion

The *H. pylori* infection was assessed by four diagnostic tests in which the prevalence was found to be 36%, taking at least two of the three diagnostic tests (RUT, culture and histology) positive while seroprevalence was 63% among the 100 patients referred for endoscopy in TUTH. RUT was found as efficient as culture and histology enhanced the chance of finding *H. pylori* in the specific sample. Statistically serological test was associated with other diagnostic tests. Prevalence of resistance was optimal for metronidazole, followed by amoxycillin, clarithromycin and tetracycline. Few multi drug resistant strains were also found in the act. Levofloxacin was found to be sensitive in all cases.

CHAPTER - VII

7. SUMMARY AND RECOMMENDATIONS

7.1 Summary

1. This study was conducted in conducted from May to October, 2009 at Public Health Research Laboratory, TUTH, Kathmandu and Norvic International Hospital and Medical college, Kathmandu with the objective to determine the prevalence of *H. pylori* infection along with antibiotic susceptibility to contemporary antibiotics. Antral biopsies were collected from 100 patients visiting endoscopy unit, TUTH.

2. The prevalence was asseseed by four diagnostic methods RUT, culture, histology and serology. Thirty two (32%) cases were positive by RUT as well as culture while 47 (47%) cases were positive by histology. The overall prevalence was 36 (36%) by considering at least two of the three diagnostic tests are positive while the seroprevalence was 63%. Serology was found associated with other three diagnostic tests statistically.

3. Among 100 patients, 55 (55%) were males and 45 (45%) were females. The male to female ratio for *H. pylori* infection was 1.22:1.

4. The prevalence of *H. pylori* infection was the highest (54.5%) in the age groups of 20 to 29 years.

5. *H. pylori* infection was higher in duodenal ulcer cases (71.4%)

6. Among 32 culture positive cases, only 30 of the bacterial isolates were subcultured and further processed for antibiotic susceptibility test. Among them 16 (53.3%) were resistant to metronidazole, 8 (26.6%) were resistant to amoxycillin, 4 (13.3%) were resistant to clarithromycin and 2 (6.6%) were resistant to tetracycline while all the isolates were susceptible to levofloxacin. 5 (16.6%) were resistant to amoxycillin and metronidazole and 2 (6.6%) were resistant to clarithromycin and metronidazole. Two (6.6%) were reistant to amoxycillin, clarithromycin, metronidazole and tetracycline.

7.2 Recommendations

This research truly manifests the present status of *H. pylori* infection in Nepal insinuating some undeniable issues;

1. At least RUT should be in regular practice in addition to histology to explore the management of *H. pylori* infection treatment.

2. Seroprevalence studies should get continuity.

3. Only with the continued surveillance of susceptibility patterns and a larger sample size of isolates will provide a more substantial answer to the issue to resistance of *H. pylori* in Nepal.

4. Levofloxacin can be a drug of choice in case of treatment failure with regular antibiotics.

5. The increasing prevalence of antibiotic resistance in *H. pylori* and falling eradication rate call for new sources of drugs and medicinal plants seem to be logical source of new antibacterial compounds.

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APPENDIX-II

Questionnaire

Date……

S.N:
Name :
Age : Sex : Male ☐ Female ☐

Food Habit : Scheduled ☐ Alcohol ☐ Smoking ☐ Other (If any)

Occupation : Work stress :

Clinical History :

Pre-Medication Used :

Endoscopic Findings :

Site of Biopsy taken :

Microbiological Study :
Rapid Urease Test :

Culture Report :

Gram Staining Result :

Biochemical Tests :

Catalase : ☐ Oxidase : ☐ Urease : ☐

Hexagon *H. Pylori* Test : ☐
(Immunochromatographic Method)

Histopathological Report:

Antibiotic Susceptibility Pattern :

APPENDIX-IV

Treatment regimens that have been repeatedly shown to be effective in eradicating *H. pylori*

Component drugs			Length of treatment (days)
Regimens based on clarithromycin			
Ranitidine, 400 mg twice daily	Clarithromycin, 500 mg twice daily		14
Ranitidine, 400 mg twice daily	Amoxicillin, 1,000 mg twice daily	Clarithromycin, 500 mg twice daily	7 to 10
Proton pump inhibitor twice daily	Amoxicillin, 1,000 mg twice daily	Clarithromycin, 500 mg twice daily	7 to 10
Regimens based on metronidazole (or tinidazole)			
Bismuth compound four times daily	Tetracycline, 500 mg four times daily	Metronidazole, 400 to 500 mg three to four times daily	14
Proton pump inhibitor twice daily	Amoxicillin, 500 mg two to three times daily	Metronidazole, 400 to 500 mg two to three times daily	7 to 10
Proton pump inhibitor twice daily	Colloidal bismuth subcitrate four times daily	Tetracycline, 500 mg four times daily	4 to 7
Regimens based on clarithromycin plus metronidazole (or tinidazole)			
Ranitidine, 400 mg twice daily	Clarithromycin, 500 mg twice daily	Metronidazole, 400 to 500 mg twice daily	7
Proton pump inhibitor twice daily	Clarithromycin, 500 mg twice daily	Metronidazole, 400 to 500 mg twice daily	7

Reprinted with permission from de Boer WA, Tytgat GN. Treatment of *Helicobacter pylori* infection. BMJ 2000; 320:31-4.

APPENDIX-V

A. Composition and preparation of different media

1. Columbia Blood Agar Base (M144)

Ingredients	**gm/liter**
Peptone, special	23 gm
Corn Starch	1 gm
Sodium chloride	5 gm
Agar	15 gm
Distilled water	1 litre
Final pH at 25ºC	7.3± 0.2

Antibiotic supplement (per vial,sufficient for 500ml medium) (FD090)

Vancomycin	5 mg
Trimethoprim	2.5 mg
Cefsulodin	2.5 mg
Amphotericin B	2.5 mg

(HiMedia Laboratories Pvt.Ltd)

Preparation of Antibiotic supplement

Components added to distilled water and brought volume to 2 ml, thoroughly mixed.

55 grams of the Columbia blood agar base medium was suspended in 1000 ml distilled water, dissolved by boiling and sterilized by autoclaving at 121^0C (15 lbs pressure) for 15 minutes. After cooling to 45-50^0C, 7% sterile defibrinated sheep blood and antibiotic supplement were added aseptically, then mixed with gentle rotation and immediately poured in sterile petriplates.

2. Blood agar base (Infusion Agar)

Ingredients	**gm/liter**
Beef heart infusion	500
Tryptose	10
Sodium chloride	5
Agar	15
Final pH at 25°C	7.3±0.2

40 grams of the blood agar base medium was suspended in 1000 ml distilled water, dissolved by boiling and sterilized by autoclaving at 121^0C (15 lbs pressure) for 15 minutes. After cooling to 45-50^0C, 7% sterile defibrinated sheep blood was added aseptically, then mixed with gentle rotation and immediately poured in sterile petriplates.
Chocolate agar was prepared by heating the blood agar at 75^0C just before the use.

3. Mueller Hinton Agar supplemented with 5% sheep blood

Ingredients	**gm/liter**
Beef infusion	300.0
Casein Hydrolysate	17.5
Starch	1.5
Agar	17.0
Final pH (at 25^0C)	7.4±0.2

38 grams of the medium was suspended in 1000 ml distilled water and the medium was warmed to dissolve completely. It was sterilized by autoclaving at 121ºC (15 lbs pressure) for 15 minutes and allowed to cool to 45-50^0C then 5% sterile defibrinated sheep blood was added to it aseptically, mixed gently and then poured into sterile petriplates.

4. Nutrient Broth

Ingredients	**gm/liter**
Peptic digest of animal tissue	5.0
Sodium chloride	5.0
Beef extract	1.5
Yeast extract	1.5
Final pH (at 25^0C)	7.4±0.2

13 grams of the medium was dissolved in 1000 ml of distilled water, boiled and dispensed into small containers. It was then sterilized by autoclaving at 121ºC (15 lbs pressure) for 15 minutes.

5. Urea broth base

Ingredients	**gm/liter**
Yeast extract	0.1
Monopotassium phosphate	9.1
Dipotassium phosphate	9.5
Phenol red	0.01
Final pH at 25°C	6.8±0.2

18.7 gram of urea broth base was suspended in 950 ml of distilled water, dissolved by boiling and sterilized by autoclaving at 121^0C (15 lbs pressure) for 15 minutes. After cooling to 55^0C, 50 ml of sterile 40% urea solution (FD048) was added aseptically, then mixed with gentle rotation. After that 0.2 ml of the urea broth was immediately dispensed into different sterile screw-capped containers.

B. Composition and preparation of different stains and reagents

1. Gram Stain

(a) Crystal violet

Solution A

Crystal violet (90% dye content)	2.0 g
Ethyl alcohol (95%)	20 ml

Solution B

Ammonium oxalate	0.8 g
Distilled Water (D/W)	80 ml

Crystal violet was dissolved in ethyl alcohol and the ammonium oxalate in D/W. Then the two solutions were mixed.

(b) Gram's Iodine

Iodine	1.0 g
Potassium Iodide	2.0 g

Distilled Water	300.0 ml

Iodine and potassium iodide was dissolved in D/W.

(c) Ethyl alcohol (95%)

Ethyl alcohol (100%)	95.0 ml
Distilled Water	5.0 ml.

(d) Safranin (Counter Stain)

Safranin (2.5% solution in 95% ethyl alcohol)	10.0 ml
Distilled Water	100.0 ml

2. Giemsa stain

Giemsa powder	3.8 g
Glycerol	250.0 ml
Methanol	250.0 ml

The Giemsa powder was weighed and transferred to a dry brown bottle. Methanol followed by glycerol as added and mixed well. The bottle was placed in a water bath at 50-60 ºC and mixed well at intervals.

3. Normal saline

Sodium Chloride	0.85 g
Distilled Water	100 ml

The sodium chloride was weighed and transferred to a leak-proof bottle premarked to hold 100 ml. Distilled water was added to the 100 ml mark, and mixed until the salt was fully dissolved. The bottle was labeled and then 0.2 ml was dipensed into sterile screw-capped containers which were then autoclaved at 121ºC (15 lbs pressure) for 15 minutes.

4. Biochemical Test Reagents

(a) Rapid Urease Test reagent

Urea solution	10 g
Phenol red solution	1g

The urea crystals was weighed and dissolved in 100 ml of distilled water to make 10 % urea solution. The phenol red powder was weighed and dissolved in 100 ml of distilled water to prepare 1 % phenol red.

(b) Catalase Reagent

Hydrogen peroxide	3 ml
Distilled Water	97 ml

To 97 ml of D/W, 3 ml of hydrogen peroxide was added and mixed well.

(c) Oxidase reagent

Oxidase discs (HiMedia Laboratories DD018)

4. McFarland tube (No. 3)

0.3 ml of 1 % $BaCl_2$ was added to 9.7 ml of 1% H_2SO_4 (v/v) with constant stirring. Then it was dispensed into a test tube labelled as 4 having the same size and volume as those used to prepare the test inoculum. The tubes were sealed tightly to prevent loss by evaporation and kept in the refrigerator. Standards may be stored for up to 6 months, after which time they should be discarded

APPENDIX-VI

Gram-staining procedure

This procedure was first devised by Hans Christian Gram during the late 19th century. It divides all bacterial species into two large groups: those that take up the basic dye, crystal violet (Gram-positive) and those that allow the crystal violet dye to wash out easily with the decolorizer alcohol or acetone (Gram-negative). It involves the following steps:

- A thin smear of the material was prepared and dried.
- The material on the slide was heat fixed and allowed to cool.
- The slide was flooded with crystal violet stain and left for 1 minute.
- It was rinsed with tap water, shaking off excess.
- It was flooded with iodine solution and left for twice as long as the crystal violet was in contact with the slide surface.
- The slide was rinsed with tap water, shaking off excess.
- The slide was flooded with decolorizer for 10 seconds and rinsed immediately with tap water
- The slide was flooded with counter-stain (safranin) for 1 minute and washed off with tap water.
- The slide was blotted between two clean sheets of bibulous paper and examined microscopically under oil immersion at 100X.

Interpretation:

Gram negative: pink

Gram positive: purple

APPENDIX-VII

Biochemical tests used for identification of bacteria

A. Catalase test

This test demonstrates the presence of catalase, an enzyme that catalyses the release of oxygen from hydrogen peroxide.

Reagent: 3% hydrogen peroxide solution.

Procedure: A pure colony from the culture plate was transferred to a clean glass slide and about 2-3 drops of 3% H_2O_2 were put on the surface of the slide. The positive test is indicated by the formation of active bubbling of the oxygen gas. A false positive reaction may be obtained if the culture medium contains catalase (e.g. Blood Agar) or if an iron wire loop is used. A control slide catalase test was performed with a small loopful of blood containing agar on the same slide. The test was considered positive if the reaction of the colony was stronger than that in the blood agar.

B. Oxidase test

This test is performed for the detection of cytochrome oxidase in bacteria which catalyzes the transport of electrons between electron donors. In the presence of redox dye Tetramethyl-*p*-phenylene diamine dihydrochloride, the cytochrome oxidase oxidizes it into a deep purple colored end product indophenol which is detected in the test.

Reagent: 1% tetramethyl-p-phenylene-diamine-dihydrochloride.

Procedure: An oxidase disc was taken and the test organism was transferred to the disc with a glass rod. The positive test was indicated by the appearance of purple colour within 10 seconds.

C. Urea Hydrolysis test: This test demonstrates the urease activity present in certain bacteria which decomposes urea, releasing ammonia and carbon dioxide. Ammonia renders the medium alkaline and thus changes the color of indicator (phenol red).

Procedure: The test organism was inoculated into the urease broth and was incubated at 37°C. A positive result was indicated by pink red colour.

APPENDIX-VIII

4.1.1 List of equipments and materials used during the study

The equipments, media, chemicals, reagents and antibiotic discs used in this study are listed below:

Equipments

Autoclave	Sakura (Japan)
Biological safety cabinet II	Dalton (Japan)
Biopsy forceps	
Endoscope	Olympus (Japan)
Hot air oven	Sakura (Japan)
Incubator	Sakura (Japan)
Microscope	Olympus (Japan)
Microtome	York Scientific Industries (India)
Refrigerator	Toshiba (Japan)
Tissue Processor	York Scientific Industries (India)
Weighing Machine	Chio (Japan)

4.1.2 Media used (Hi-Media, India)

Blood Agar

Columbia Blood Agar Base

Mueller Hinton Agar

Nutrient Broth

Urea Broth Base

4.1.3 Chemicals and reagents

3% Hydrogen peroxide

40% Urea Solution

Absolute (95%) alcohol

Crystal Violet

D.P.X Mountant

Gram's Iodine

Normal saline

Safranin

4.1.4 Antibiotic discs

Hi Media

Amoxycillin (10 mcg)

Clarithromycin (15 mcg)

Levofloxacin (5mcg)

Metronidazole (5 mcg)

Tetracycline (30 mcg)

4.1.5 Other disc

Oxidase disc

4.1.6 Miscellaneous

Conical flasks, Cotton, Distilled water, Micropipettes, Forceps, Glass slides, Immersion oil, Inoculating loop, Glass rods, Lysol, Measuring cylinder, Petri dishes, Pipettes, Glass containers, Spatula, Test tubes, Wooden applicators, Sheep blood, Candle.

APPENDIX-IX

Statistical analysis (Chi-square test)

Association of serology and other diagnostic tests

Other Than Serology (Culture, Histology & RUT)	Serology		Total
	Positive	Negative	
Positive	28 (a)	8 (b)	36 (a+b)
Negative	35 (c)	29 (d)	64 (c+d)
Total	63 (a+c)	37 (b+d)	100 (a+b+c+d)

The test statistic is χ^2

Null hypothesis (H_0): There is no association between serology and other diagnostic tests.

Alternate hypothesis (H_1): There is an association between serology and other diagnostic tests.

From

$$\chi^2 = \frac{(ad-bc)^2 (a+b+c+d)}{(a+b)(c+d)(a+c)(b+d)} = 5.269$$

Degree of freedom = (2-1) (2-1)

From table, χ^2 at 5 % level of significance = 3.841

Thus $\chi^2_{cal} > \chi^2_{tab}$

Hence, H_1 is accepted i.e. there is significant association between serology and other diagnostic tests.

APPENDIX-II

Questionnaire

Date……

S.N:
Name :
Age : Sex : Male ☐ Female ☐

Food Habit : Scheduled ☐ Alcohol ☐ Smoking ☐ Other (If any)

Occupation : Work stress :

Clinical History :

Pre-Medication Used :

Endoscopic Findings :

Site of Biopsy taken :

Microbiological Study :
Rapid Urease Test :

Culture Report :

Gram Staining Result :

Biochemical Tests :

Catalase : ☐ Oxidase : ☐ Urease : ☐

Hexagon *H. Pylori* Test : ☐
(Immunochromatographic Method)

Histopathological Report:

Antibiotic Susceptibility Pattern :

APPENDIX-IV

Treatment regimens that have been repeatedly shown to be effective in eradicating *H. pylori*

Component drugs			Length of treatment (days)
Regimens based on clarithromycin			
Ranitidine, 400 mg twice daily	Clarithromycin, 500 mg twice daily		14
Ranitidine, 400 mg twice daily	Amoxicillin, 1,000 mg twice daily	Clarithromycin, 500 mg twice daily	7 to 10
Proton pump inhibitor twice daily	Amoxicillin, 1,000 mg twice daily	Clarithromycin, 500 mg twice daily	7 to 10
Regimens based on metronidazole (or tinidazole)			
Bismuth compound four times daily	Tetracycline, 500 mg four times daily	Metronidazole, 400 to 500 mg three to four times daily	14
Proton pump inhibitor twice daily	Amoxicillin, 500 mg two to three times daily	Metronidazole, 400 to 500 mg two to three times daily	7 to 10
Proton pump inhibitor twice daily	Colloidal bismuth subcitrate four times daily	Tetracycline, 500 mg four times daily	4 to 7
Regimens based on clarithromycin plus metronidazole (or tinidazole)			
Ranitidine, 400 mg twice daily	Clarithromycin, 500 mg twice daily	Metronidazole, 400 to 500 mg twice daily	7
Proton pump inhibitor twice daily	Clarithromycin, 500 mg twice daily	Metronidazole, 400 to 500 mg twice daily	7

Reprinted with permission from de Boer WA, Tytgat GN. Treatment of *Helicobacter pylori* infection. BMJ 2000; 320:31-4.

APPENDIX-V

A. Composition and preparation of different media

1. Columbia Blood Agar Base (M144)

Ingredients	**gm/liter**
Peptone, special	23 gm
Corn Starch	1 gm
Sodium chloride	5 gm
Agar	15 gm
Distilled water	1 litre
Final pH at 25ºC	7.3± 0.2

Antibiotic supplement (per vial,sufficient for 500ml medium) (FD090)

Vancomycin	5 mg
Trimethoprim	2.5 mg
Cefsulodin	2.5 mg
Amphotericin B	2.5 mg

(HiMedia Laboratories Pvt.Ltd)

Preparation of Antibiotic supplement

Components added to distilled water and brought volume to 2 ml, thoroughly mixed.

55 grams of the Columbia blood agar base medium was suspended in 1000 ml distilled water, dissolved by boiling and sterilized by autoclaving at 121^0C (15 lbs pressure) for 15 minutes. After cooling to 45-50^0C, 7% sterile defibrinated sheep blood and antibiotic supplement were added aseptically, then mixed with gentle rotation and immediately poured in sterile petriplates.

2. Blood agar base (Infusion Agar)

Ingredients	**gm/liter**
Beef heart infusion	500
Tryptose	10
Sodium chloride	5
Agar	15
Final pH at 25°C	7.3±0.2

40 grams of the blood agar base medium was suspended in 1000 ml distilled water, dissolved by boiling and sterilized by autoclaving at 121^0C (15 lbs pressure) for 15 minutes. After cooling to 45-50^0C, 7% sterile defibrinated sheep blood was added aseptically, then mixed with gentle rotation and immediately poured in sterile petriplates.
Chocolate agar was prepared by heating the blood agar at 75^0C just before the use.

3. Mueller Hinton Agar supplemented with 5% sheep blood

Ingredients	**gm/liter**
Beef infusion	300.0
Casein Hydrolysate	17.5
Starch	1.5
Agar	17.0
Final pH (at 25^0C)	7.4±0.2

38 grams of the medium was suspended in 1000 ml distilled water and the medium was warmed to dissolve completely. It was sterilized by autoclaving at 121ºC (15 lbs pressure) for 15 minutes and allowed to cool to 45-50^0C then 5% sterile defibrinated sheep blood was added to it aseptically, mixed gently and then poured into sterile petriplates.

4. Nutrient Broth

Ingredients	**gm/liter**
Peptic digest of animal tissue	5.0
Sodium chloride	5.0
Beef extract	1.5
Yeast extract	1.5
Final pH (at 25^0C)	7.4±0.2

13 grams of the medium was dissolved in 1000 ml of distilled water, boiled and dispensed into small containers. It was then sterilized by autoclaving at 121ºC (15 lbs pressure) for 15 minutes.

5. Urea broth base

Ingredients	**gm/liter**
Yeast extract	0.1
Monopotassium phosphate	9.1
Dipotassium phosphate	9.5
Phenol red	0.01
Final pH at 25°C	6.8±0.2

18.7 gram of urea broth base was suspended in 950 ml of distilled water, dissolved by boiling and sterilized by autoclaving at 121^0C (15 lbs pressure) for 15 minutes. After cooling to 55^0C, 50 ml of sterile 40% urea solution (FD048) was added aseptically, then mixed with gentle rotation. After that 0.2 ml of the urea broth was immediately dispensed into different sterile screw-capped containers.

B. Composition and preparation of different stains and reagents

1. Gram Stain

(a) Crystal violet

Solution A

Crystal violet (90% dye content)	2.0 g
Ethyl alcohol (95%)	20 ml

Solution B

Ammonium oxalate	0.8 g
Distilled Water (D/W)	80 ml

Crystal violet was dissolved in ethyl alcohol and the ammonium oxalate in D/W. Then the two solutions were mixed.

(b) Gram's Iodine

Iodine	1.0 g
Potassium Iodide	2.0 g
Distilled Water	300.0 ml

Iodine and potassium iodide was dissolved in D/W.

(c) Ethyl alcohol (95%)

Ethyl alcohol (100%)	95.0 ml
Distilled Water	5.0 ml.

(d) Safranin (Counter Stain)

Safranin (2.5% solution in 95% ethyl alcohol)	10.0 ml
Distilled Water	100.0 ml

2. Giemsa stain

Giemsa powder	3.8 g
Glycerol	250.0 ml
Methanol	250.0 ml

The Giemsa powder was weighed and transferred to a dry brown bottle. Methanol followed by glycerol as added and mixed well. The bottle was placed in a water bath at 50-60 ºC and mixed well at intervals.

3. Normal saline

Sodium Chloride	0.85 g
Distilled Water	100 ml

The sodium chloride was weighed and transferred to a leak-proof bottle premarked to hold 100 ml. Distilled water was added to the 100 ml mark, and mixed until the salt was fully dissolved. The bottle was labeled and then 0.2 ml was dipensed into sterile screw-capped containers which were then autoclaved at 121ºC (15 lbs pressure) for 15 minutes.

4. Biochemical Test Reagents

(a) Rapid Urease Test reagent

Urea solution	10 g
Phenol red solution	1g

The urea crystals was weighed and dissolved in 100 ml of distilled water to make 10 % urea solution. The phenol red powder was weighed and dissolved in 100 ml of distilled water to prepare 1 % phenol red.

(b) Catalase Reagent

Hydrogen peroxide	3 ml
Distilled Water	97 ml

To 97 ml of D/W, 3 ml of hydrogen peroxide was added and mixed well.

(c) Oxidase reagent

Oxidase discs (HiMedia Laboratories DD018)

4. McFarland tube (No. 3)

0.3 ml of 1 % $BaCl_2$ was added to 9.7 ml of 1% H_2SO_4 (v/v) with constant stirring. Then it was dispensed into a test tube labelled as 4 having the same size and volume as those used to prepare the test inoculum. The tubes were sealed tightly to prevent loss by evaporation and kept in the refrigerator. Standards may be stored for up to 6 months, after which time they should be discarded

APPENDIX-VI

Gram-staining procedure

This procedure was first devised by Hans Christian Gram during the late 19th century. It divides all bacterial species into two large groups: those that take up the basic dye, crystal violet (Gram-positive) and those that allow the crystal violet dye to wash out easily with the decolorizer alcohol or acetone (Gram-negative). It involves the following steps:

- A thin smear of the material was prepared and dried.
- The material on the slide was heat fixed and allowed to cool.
- The slide was flooded with crystal violet stain and left for 1 minute.
- It was rinsed with tap water, shaking off excess.
- It was flooded with iodine solution and left for twice as long as the crystal violet was in contact with the slide surface.
- The slide was rinsed with tap water, shaking off excess.
- The slide was flooded with decolorizer for 10 seconds and rinsed immediately with tap water
- The slide was flooded with counter-stain (safranin) for 1 minute and washed off with tap water.
- The slide was blotted between two clean sheets of bibulous paper and examined microscopically under oil immersion at 100X.

Interpretation:

Gram negative: pink

Gram positive: purple

APPENDIX-VII

Biochemical tests used for identification of bacteria

A. Catalase test

This test demonstrates the presence of catalase, an enzyme that catalyses the release of oxygen from hydrogen peroxide.

Reagent: 3% hydrogen peroxide solution.

Procedure: A pure colony from the culture plate was transferred to a clean glass slide and about 2-3 drops of 3% H_2O_2 were put on the surface of the slide. The positive test is indicated by the formation of active bubbling of the oxygen gas. A false positive reaction may be obtained if the culture medium contains catalase (e.g. Blood Agar) or if an iron wire loop is used. A control slide catalase test was performed with a small loopful of blood containing agar on the same slide. The test was considered positive if the reaction of the colony was stronger than that in the blood agar.

B. Oxidase test

This test is performed for the detection of cytochrome oxidase in bacteria which catalyzes the transport of electrons between electron donors. In the presence of redox dye Tetramethyl-*p*-phenylene diamine dihydrochloride, the cytochrome oxidase oxidizes it into a deep purple colored end product indophenol which is detected in the test.

Reagent: 1% tetramethyl-p-phenylene-diamine-dihydrochloride.

Procedure: An oxidase disc was taken and the test organism was transferred to the disc with a glass rod. The positive test was indicated by the appearance of purple colour within 10 seconds.

C. Urea Hydrolysis test: This test demonstrates the urease activity present in certain bacteria which decomposes urea, releasing ammonia and carbon dioxide. Ammonia renders the medium alkaline and thus changes the color of indicator (phenol red).

Procedure: The test organism was inoculated into the urease broth and was incubated at 37°C. A positive result was indicated by pink red colour.

APPENDIX-VIII

4.1.1 List of equipments and materials used during the study

The equipments, media, chemicals, reagents and antibiotic discs used in this study are listed below:

Equipments

Autoclave	Sakura (Japan)
Biological safety cabinet II	Dalton (Japan)
Biopsy forceps	
Endoscope	Olympus (Japan)
Hot air oven	Sakura (Japan)
Incubator	Sakura (Japan)
Microscope	Olympus (Japan)
Microtome	York Scientific Industries (India)
Refrigerator	Toshiba (Japan)
Tissue Processor	York Scientific Industries (India)
Weighing Machine	Chio (Japan)

4.1.2 Media used (Hi-Media, India)

Blood Agar

Columbia Blood Agar Base

Mueller Hinton Agar

Nutrient Broth

Urea Broth Base

4.1.3 Chemicals and reagents

3% Hydrogen peroxide

40% Urea Solution

Absolute (95%) alcohol

Crystal Violet

D.P.X Mountant

Gram's Iodine

Normal saline

Safranin

4.1.4 Antibiotic discs

Hi Media

Amoxycillin (10 mcg)

Clarithromycin (15 mcg)

Levofloxacin (5mcg)

Metronidazole (5 mcg)

Tetracycline (30 mcg)

4.1.5 Other disc

Oxidase disc

4.1.6 Miscellaneous

Conical flasks, Cotton, Distilled water, Micropipettes, Forceps, Glass slides, Immersion oil, Inoculating loop, Glass rods, Lysol, Measuring cylinder, Petri dishes, Pipettes, Glass containers, Spatula, Test tubes, Wooden applicators, Sheep blood, Candle.

APPENDIX-IX

Statistical analysis (Chi-square test)

Association of serology and other diagnostic tests

Other Than Serology (Culture, Histology & RUT)	Serology		Total
	Positive	Negative	
Positive	28 (a)	8 (b)	36 (a+b)

Negative	35 (c)	29 (d)	64 (c+d)
Total	63 (a+c)	37 (b+d)	100 (a+b+c+d)

The test statistic is χ^2

Null hypothesis (H_0): There is no association between serology and other diagnostic tests.

Alternate hypothesis (H_1): There is an association between serology and other diagnostic tests.

From

$$\chi^2 = \frac{(ad-bc)^2(a+b+c+d)}{(a+b)(c+d)(a+c)(b+d)} = 5.269$$

Degree of freedom = (2-1) (2-1)

From table, χ^2 at 5 % level of significance = 3.841

Thus $\chi^2_{cal} > \chi^2_{tab}$

Hence, H_1 is accepted i.e. there is significant association between serology and other diagnostic tests.

Photograph 4 Colonies of *H. pylori* on Blood agar plate

Photograph 5 Gram staining of *H. pylori*

Photograph 6 Giemsa staining of *H. pylori*

Photograph 7 Antibiotic susceptibility test of *H. pylori*