Chapter One

Introduction

Discovery of *Helicobacter* organism was considered one of the most significant advances in gastrointestinal pathology during 20th century; the organism was thought originally to be a member of the genus *Campylobacter* and was named *Campylobacter pyloridis*. Later it was corrected to *Campylobacter pylori*: subsequent 16S rRNA sequence analysis showed that the distance between the true *Campylobacter* and *C.pylori* was sufficient to exclude it from the *Campylobacter* genus (Fox *et al*., 1992).

taxonomy in group 2 which includes aerobic microaerophilic spine, helical and curved rod Gram negative bacteria (Holt *et al* 1994). *H.pylori* includes its family *Helicobacteraceae*. *H.pylori* is a typical curved rod – like or short spiral (up to three turns) (Morris *et al*., 1990). The bacterium changes its morphology from a helical form to coccoid under various conditions such as extended cultivation, aerobic culture, alkaline pH and antibiotic treatment (Catrenick and Makin, 1991).

Studying modes of transmission of *H.pylori* is difficult, although many researches have been devoted to determine how *Helicobacter* infection

is acquired. Current evidence indicates that milks and gastric tissue from Sardinia sheep were cultured and analyzed by PCR and *Helicobacter pylori* was found in 60% (38/63) of milk samples and 30% (6/20) of sheep tissue samples (Dore, 1999).

Diagnostic tests for *H.pylori* are categorized as either direct (non invasive) serological and urea breath test, or indirect (invasive) includes rapid urease test, histology and culture methods (Hopkins and Morris, 1994). No single technique is perfect for the diagnosis of *H.pylori*, therefore, a combination of different tests must be performed to obtain best results.

The need for new strategies to eradicate *H.pylori*, alternative or complementary to antibiotic therapy, has recently claimed the attention of many investigators.

It was reported that *Lactobacilli* can inhibit the growth of *H.pylori* in vitro and exhibits antagonistic activity against it (Kabir *et al.*, 1997). In human volunteers, the spent culture supernatant of human *Lactobacillus acidophilus* strain LA1 is active against *H.pylori* possibly due to the production of lactic acid or to the secretion of antibiotic (Michetti *et al.*, 1995).

Aims of the Study:

1- Isolation of *Helicobacter pylori* from human and some domestic animals.
2- Characterization of *Helicobacter pylori* isolates.
3- Investigate the correlation between *Helicobacter pylori* from human and some domestic animal origins, with emphasis on the possibility of its transmission from such animals to human.
4- Histological examination of *Helicobacter pylori* in both human and animal tissues.
5- Studying the susceptibility of *H.pylori* to antimicrobial agents.
6- Studying inhibitory effect of *L.acidophilus* on the adhesion property of *H.pylori*.

Chapter Two

Literature Review

2. Literature Review

2.1 Discovery of *Helicobacter*:

Helicobacter pylori was considered to be pathogen even before the early observations of gastric spiral bacteria in humans. Similar organisms were seen in animals in 1881 in a thesis submitted to the Faculty of Medicine describing spiral bacteria in gastric scrapings from dogs. This observation was later confirmed by performing experimental inoculations with gastric scrapings from dogs. Similar organisms were subsequently seen in cats, rhesus macaques and more recently in a variety of other animals (Fox and Lee, 1997; Solnick and Schauer, 2001).

It was long believed that the stomach was sterile or colonized only with small numbers of bacteria. The mechanism of what was referred to as the "gastric bactericidal barrier" was debated in the early part of the 20th century. But most authors then, as well as, more recently concluded that the predominant effect was due to gastric acid. However, cultivation of a novel bacterium from gastric mucosa in 1982 marked a turning point in ourstanding of gastrointestinal microbial ecology and disease (Dooley and Cohen, 1988).

Marshall and Warren (1984) described spiral or curved bacilli in histological sections from 58 of 100 consecutive biopsy specimens of human gastric antral mucosa, 11 of which were culture positive for Gram negative, microaerophilic bacterium. The organism was thought originally to be a member of the genus *Campylobacter* and was

named *Campylobacter pyloridis*, later corrected to *Campylobacter pylori*. Because subsequent 16S rRNA sequence analysis showed that the distance between the true *Campylobacter* and *C.pylori* was sufficient to exclude it from the *Campylobacter* genus (Fox *et al*., 1992), it was renamed *H.pylori*. The first member of the new genus was *Helicobacter* (Goodwin *et al*., 1989). Nevertheless, Marshall and Warren (1984) were not the first to detect gastric spiral bacteria spiral organisms; they were first seen in human gastric mucosa in the beginning of the 20th century. The bacteria were often seen in malignant or ulcerated gastric tissues and the possibility of an infectious cause of peptic ulcer disease was considered (Hentschel *et al*., 1993; Sonneberg, 1998). Some investigators have specifically proposed that a search be made for an organism "thriving in [illegible] as a possible factor of chronicity, if not an etiological factor, in [illegible] (Quirk, 1986; Baron [illegible] by National Institutes of Health consensus panel that recommended antibiotic therapy for long majority of peptic ulcer patients who are infected with *H.pylori* and by classification of *H.pylori* as a class I (definite) carcinogen by the World Health Organization (Marshall, 1994).

At least 16 and probably 18 species of *Helicobacter* have been isolated and identified from the stomach and intestine of various animals, including dogs, cats, ferrets, minks, pigs, monkeys, sheep, mice, rats, hamsters, cheetahs and birds (Fox and Lee, 1997). Bacteria that resemble *H.pylori* have been also found frequently in the bovine abomassum (Solnick and Schaur, 2001).

2.2 Morphological Observation of *Helicobacter*:

Most early observations on gastric spiral bacteria were made in dogs and cats. When the first electron micrograph of this bacterium was published, it was immediately apparent that more than one morphological form could be found (Weber and Schmitted, 1962). Three morphologic forms of these organisms in dogs have been reported and classified according to Lockard and Bolar type methods based on morphological criteria, including the length, width, coils, periplasmic fibers and sheathed flagella. The first quality electron micrographs of what are now called Lockard type 1, 2 and 3 bacteria are all now known to represent *Helicobacter* species (Lockard and Bolar 1979).

Lockard type 1, which is representative of species *Helicobacter flexispira*, *Helicobacter bilis* and the enterohepatic *Helicobacter* [illegible] tightly spiral and multiple periplasmic fibers appear to cover the entire surface of the bacterium. Lockard type 2 is spiral rather than cylindrical and has periplasmic fibers that are more sparsely distributed and can appear singly or in groups of two, three and occasionally four. This organism is the typical morphology of *Helicobacter felis*. Lockard type 3, which resembles type 2 but is somewhat more tightly coiled and does not have periplasmic fibers, is typical of *Helicobacter bizzozeronii* and the un - cultivated *"Helicobacter heilmanii"*. A fourth type, similar to Lockard type 3 but thicker and with fewer coils, was described by Weber and Schmitted (1962) but not Lockard and Bolar this organism may represent *Helicobacter salomonis*, recently cultivated from dogs.

2.3 Taxonomy of *Helicobacter*:

Many spirals Gram negative bacteria isolated from the mammalian gastrointestinal tract were grouped as *Campylobacter*. This classification was based on similar microscopic Gram stain morphologic, common microaerophilic growth requirements and similar ecologic niche (Table 2-1). Ultrastructural differences such as the presence of sheathed flagella in *Helicobacter* organisms provided clues that distinguished *Helicobacter* organisms from *Campylobacter*. Moreover, partial sequencing of 16S rRNA genes yielded evidence that *Campylobacter* belonged to a different genus (Romaniuk *et al*., 1987).

The genus *Helicobacter* was formerly distinguished from other Gram negative curved rods (e.g. *Campylobacter*) follow[illegible] extensive [illegible] biochemical activities, fatty acid profiles, growth characteristics, nucleic acid hybridization profiles [illegible]

2.4 Gastric *Helicobacter* Species:

Since *H.pylori* was first cultivated from human gastric biopsy specimens in 1982, it has become apparent that many related species can often be found colonizing the mucosal surface of humans and other animals (Fox, 1997; Solnick and Schaur, 2001). These other *Helicobacter* species can be broadly grouped according to whether they colonize the gastric or enterohepatic niche (Diwan *et al*., 1997).

Table (2-1): Habitats and Phenotypic Characteristics of *Helicobacter* Species (Fox and – Lee, 1997).

Helicobacter taxon	Source (s)	Primary Site	Catalase production	Nitrate reduction	Alkaline Phosphatase	Urease	Indoxyl Acetate Hydrolysis	γ Glutamyl transferase	Growth At 24° C	Growth With 1% glycine	Resistance to[b]: Nalidixic Acid	Resistance to[b]: Cephalothin	Flagella
Human													
H. bizzozeroniif	**Human, dog**	**Stomach**	+	+	+	+	+	+	+	-	**R**	**S**	**Bipolar**
H.canis	**Human, dog**	**Intestine**	-	-	+	-	+	ND	+	ND	**S**	**I**	**Bipolar**
H.cinaedi	**Human, Bamster**	**Intestine**	+	+	-	-	-	-	-	+	**S**	**I**	**Bipolar**
H.femelliae	**Human**	**Intestine**	-	-	+	-	+	-	-	+	**S**	**S**	**Bipolar**
H.pullonan	**chicken**	**Intestine**	-	-	+	-	+	-	-	+	**S**	**S**	**Monopolar**
H.Pylori	**Human, Macaque**	**Stomach**	+	-	+	+	-	-	-	-	**R**	**S**	**Monopolar**
H.westmeadii	**Human**	**Unknown**	+	+	-	-	ND	ND	-	ND	**S**	**R**	**Monopolar**
Nonhuman													
H.acinonyx	**Cheetah**	**Stomach**	+	+	+	+	-	-	+	-	**R**	**S**	**Bipolar**
H.bilis	**Mouse**	**Intestine**	-	-	ND	+	+	ND	-	+	**R**	**R**	**Bipolar**
H.cholecystus	**Hamster**	**Intestine**	+	+	+	+	+	+	-	-	**R**	**R**	**Bipolar**
H.felis	**Cat**	**Stomach**	+	+	+	+	-	+	+	-	**R**	**S**	**Bipolar**
H.hepaticus	**Mouse**	**Intestine**	+	+	ND	+	+	ND	-	+	**R**	**R**	**Bipolar**
H.muridarum	**Mouse , rat**	**Intestine**	+	-	+	+	+	+	-	-	**R**	**R**	**Bipolar**
H.mustelae	**Ferret , mink**	**Stomach**	+	+	+	+	+	+	+	-	**S**	**R**	**peritrichous**
H.nemestrinae	**Macaque**	**Stomach**	+	-	+	+	-	ND	+	-	**R**	**S**	**Bipolar**
H.panetensis	**Birds , swine**	**Intestine**	+	+	+	-	-	-	+	+	**S**	**S**	**Bipolar**
H.rodenttium	**Mouse**	**Intestine**	+	+	-	-	-	-	+	+	**R**	**R**	**Bipolar**
H.salomonis	**Dog**	**Stomach**	+	+	+	+	+	+	-	**ND**	**R**	**S**	**Bipolar**
H.trogontum	**Rat**	**Intestine**	+	+	-	+	**ND**	+	+	**ND**	**R**	**R**	**Bipolar**
"H.rappini"	**Human ,dog, Sheep , mouse**	**Intestine**	+	-	-	+	**ND**	+	+	-	**R**	**R**	**Bipolar**

The *Helicobacter* species that infect humans can be divided into two types: the gastric, urease producers (*H.pylori* and *Helicobacter heilmanni* [also known as *Gastrospirillum hominis*]), and the enteric non urease producers (*Helicobacter cinaedi* and *Helicobacter fennelliae* [formerly *Campylobacter cinaedi* and *Campylobacter fennelliae*]) (Orlicak *et al*., 1993).

Other *Helicobacter* species are widely distributed in mammalian hosts and are often nearly universally prevalent. In many cases, they cause an inflammatory response resembling that seen with *H.pylori* in humans. Although usually not pathogenic in their natural host (Dick and Lee, 1991), a case report of human infection with three *Helicobacter* species and infected humans (*Helicobacter pullorum*, *Helicobacter* and *Helicobacter cannis*) have not been fully enough substantiated

2.5 *Helicobacter pylori*:

Helicobacter pylori is a spiral, Gram negative bacterium which inhibits the stomach of more than (50%) of human, although the new organism was only cultured in 1982 (Marshall and Warren 1984). Its manifestations have been reported in the scientific literature for over 100 years. When dogs harbored a "spirochete" bacteria that could survive in the acid secreting stomach. Other investigators noticed that urease was usually present in the stomach of carnivorous animals such as dogs and cats and these observations were later extended to human. Before it was thought that all these observations were unrelated and that gastric urease was actually secreted by the gastric epithelial cells, until Lieber and Lefevre in 1959 showed that it could be suppressed with tetracycline, while Delluva *et al.* in 1968 showed that germ - free animals did not develop gastric urease. The presence of spiral bacteria in the human stomach was reported most notably by Friedberg [illegible] and Steer, [illegible] were found to cause peptic ulcer and gastric cancer (Marshall and Warren, 1984; Marshall, 2000).

H.pylori has repeatedly been shown to be associated with chronic superficial gastritis (CSG) which involves the antrum and the fundus of the stomach (Genta *et al.*, 1994). Chronic gastritis is well known factor for the development of gastric carcinoma. In addition, the development of intestinal metaplasia and atrophic gastritis, two risk factors for gastric cancer, are associated with *H.pylori* infection .These organisms are present on the animal surface of mucus - secreting cells within gastric pets but do not invade tissue. Colonization of the affected areas may be adjacent to those with no colonization. Organisms are generally not present over areas of intestinal metaplasia in the gastric mucosa (Blasar and Parsonnet 1994; Mohammed, 2004).

2.5.1 General Characteristics of *Helicobacter pylori*:-

H.pylori are classified according to Bergeis manual taxonomy in group 2 which includes aerobic, microaerophilic, motile, helical and curved rod, Gram negative bacteria (Holt *et al*., 1994). This classification also includes its family *Helicobacteraceae*.

The organism is approximately (0.6µm) wide and (3.5µm) long taking the shape of a spiral in tissue section and biopsy smears it appears either curved, gently spiral bacteria or S - shaped. While, on cultivation true spiral forms may be few or absent and the organism appears mostly as bacilli or slightly curved (Blaser, 1990).

H.pylori are motile, even in the highly viscous mucous layer in which they live. The organism has up to 7 sheathed flagella attached to one pole which allow for motility through propelling itself with a rapid corkscrew like movement that allows it to penetrate the mucous gel layer overlaying the gastric epithelium (Hyg *et al*., 1993). [illegible] like or short spiral (up to th[illegible]

Anderson *et al*., (1990) reported that in vitro growth in broth media with shaking can induce *H.pylori* to assume long spiral morphology with greater than 5 turns, while curved rods and short spiral organisms were seen when growing on solid media like blood agar. In addition, the bacterium changes its morphology from a helical form to coccoid under various conditions such as extended cultivation, aerobic culture, alkaline pH and antibiotic treatment (Catrenick and Makin 1991; Suberg *et al*, 1996).

The addition of either ferrus sulfate and sodium pyruvate or mucin to Brain heart infusion broth with (7%) horse serum (BHI-HS) enhanced growth of the bacteria (Jiang and Doyle, 2000). Slow growing bacteria can be inhibited by addition of appropriate antibiotics to make the media used for *H.pylori* selective isolation (Collee *et al*., 1996).

Appropriate transport conditions of the biopsy are important in order to avoid desiccation of biopsy samples and long exposure to ambient air (Megraud *et al*., 1997).

2.5.2 Epidemiology:-

There is very little information on actual modes of transmission of this organism, but its presence in the stomach suggests that *H.pylori* may be food or water borne (from fecal contamination). It may also be expelled during vomiting and then, under unhygienic conditions be acquired by new host (Ellin, 1996).

Transmission of *H.pylori* is likely to occur by multiple routes affected by local conditions and behavior (Feldman, 1995). Close person to person contact seems to enhance the transmission by oral - oral or fecal - oral routes, [illegible] (Parsonnet and Blaser, 1994; Fox *et al*., 1996).

2.5.3 Prevalence in Healthy Individual:-

Human *H.pylori* has world wide distribution and its prevalence in healthy and a symptomatic person is (15 to 10)% depending on age, socioeconomic class and country origin (Megraud, 1993).

Axon, (1995) suggested that since *H.pylori* doesn't cause diarrhea, it is probably not spread by fecal - oral route. Rather he proposes that *H.pylori* facilities its transmission to new host by inducing an upset stomach and vomiting in children, resulting in the spread of infection in crowded conditions where sanitation is inadequate.

It was found that infection is usually acquired in childhood, and in developing countries. Children are typically infected by the age 10 years whereas in developed countries there is an age related increase in prevalence. This was due to a steady falling rate of acquisition of the infection as well as loss of the infection possibly owing to the widespread use of antibiotics (Sipponen *et al*., 1996).

Therefore, epidemiological studies have demonstrated a correlation between colonization and age, low socio - economic status and over crowding, particularly during childhood (Webb *et al.*, 1994; Peterson and Graham, 2001). The explanation for these observations has included environment and host genetic component, which will be briefly discussed as follows:

2.5.3.1 Environmental Factors:

Acquisition of *H.pylori* infection occurs predominantly during childhood with acquisition during adulthood occurring at a rate of only (0.3 to 0.5)% a year (Cullen *et al.*, 1993). Major risk factor for infection is the socioeconomic status of the family during childhood as reflected in the number of persons in a household, sharing a bed, and absence of a fixed hot water supply. All of which are probably markers for the level of sanitation and household hygiene (Webb *et al.*, 1994). The age related apparent increase the prevalence of the infection in developed countries can best be explained by the birth cohort effect. As successive generation has been less likely to become infected as children. These cohorts show lower frequency of infection as adults (Peterson and Graham, 2001).

2.5.3.2 Genetic Factors:

Genetic susceptibility to infection has been confirmed in studies showing that monozygotic twins reared a part or together had a high rate of concordance of infection than did age - matched dizygotic twin (Malaty *et al.*, 1994). Another study confirmed older data showing a genetic effect in the *H.pylori* - related disease, peptic ulcer disease (Malaty *et al.*, 2000).

2.5.4 Prevalence in Animals:-

Studying modes of transmission of *H.pylori* is difficult, although some research has been devoted to determine how *Helicobacter* infection [illegible] acquisition (Cullen *et al.*, 1993). Current evidence indicates that milks and gastric tissue from Sardinia sheep were cultured and analyzed by PCR and [illegible] 0% (38/63) [illegible] of sheep tissue samples. These organisms were cultured from sheep milk and tissue samples and confirmed as *H.pylori* based on (sequences analysis of 16S rRNA PCR). The presence of *H.pylori* like bacteria and the fact that gastritis was absent or at most mild argues that sheep may be a natural host for *H.pylori* (Dore *et al.*, 1999).

The organism has been cultured from a colony of research cats (Handt *et al.*, 1994), but not been found in stray cats (El-Zaatary *et al*, 1997). Handt *et al.*, (1994) observed that *H. pylori* can be isolated from cats and suggests transmission from pets to humans or from humans to pets is also possible. A study reporting *H. pylori* in commercial vector cats led to a suggestion that *H. pylori* may be zoontic pathogen with transmission occurring from cats to humans (El-Zaatary *et al.*, 1997).

A study done by Bohmler *et al.*, (1996) examined 177 samples of udder secretion from cows with mastitis, 199 samples of milk from healthy cows, and 100 chicken stomachs. Result showed that none of them contained *H.pylori*. Also *H.pylori* cells survived for up to several weeks in drip water from a thawed chicken which had been frozen at (-20°C). Such finding indicate that fresh milk and chicken are not likely to contain *H.pylori*, but that if these food were contaminated because of inadequate hygienic, the bacteria may survive enough to cause infection.

Iatrogenic transmission via biopsy forceps or endoscope is also possible when these instruments were not properly cleaned and disinfected (Fantry *et al.*, 1995). Also infection of endoscope staff would suggest the main route for respiratory tract infection. The inhaled organisms would presumably

2.5.3 Virulence of *H.pylori*

H.pylori may be

(Peterson and Graham, 2001).

Table (2-2): Virulence Factors of *H.pylori* that Promote Colonization and Induce Tissue Injury (Peterson and Graham, 2001).

Promote Colonization	Induce Tissue Injury
Flagella (for motility).	Lipopolysacchride.
Urease.	Leukocyte recruitment and activating factor.
Adherence factor.	Vacuolating cytotoxin (VacA).
	Cytotoxin associated antigen (Cag A).
	Outer membrane inflammatory protein (Oip A).
	Heat shock proteins (HsPA, HsPB).

2.5.5.1 Colonization Factors:

Colonization factors are those attributes of *H.pylori* that allow it to establish its presence in the stomach and to persist the body's attempts to rid itself of infection. These factors are:-

A- Motility

Motility of *H.pylori* is essential for colonization, it allows the bacteria to spread through the viscous mucus covering the epithelial cells of gastric mucosa (Hazell *et al.*, 1986).

The property of these flagella is the presence of sheath covering the flagella filament. This sheath composed of a double layer of phospholipids and is thought to protect the flagella from the gastric acidity, which otherwise would depolymerize flagella filaments (Geies *et al.*, 1993). Jones *et al.*, protein, which has been reported to an N - acetylneuraminyl lactos a hemagglutinin. Unlike other bacterial flagella filaments, which are *H.pylori* B (Leying *et al.*, 1992).

B- Urease:

H.pylori is more powerful producer of urease than almost any other bacterial species. This enzyme, a 300 to 625 KD a nickel containing hexamer, is located in the cell membrane and is also actively excreted into the gastric lumen (Lee *et al.*, 1993). Urease is essential for *H.pylori* colonization of the stomach but not required for survival after colonization (Eaton *et al.*, 1991; Lee *et al.*, 1993). By hydrolyzing urea to carbon dioxide and ammonia, urease may surround organism with a cloud of ammonia, protecting it from the stomach's acid environment as described in (Figure 2-1) (Marshall *et al.*, 1987).

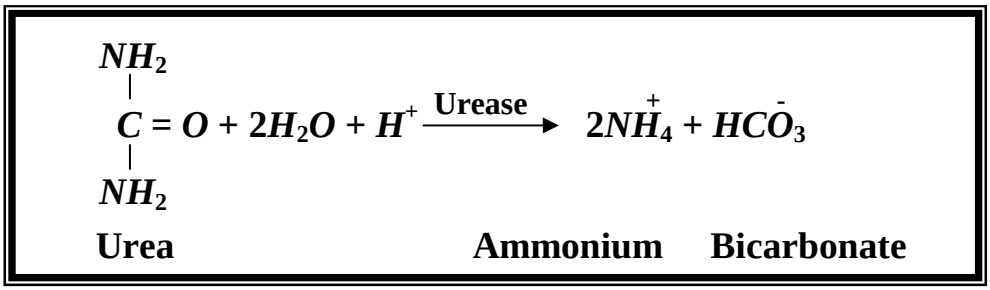


Figure (2-1): Cleavage of urea by urease enzyme (Marshall *et al.*, 1987).

High molecular weight enzyme demonstrates much higher affinity for substrate and significantly higher activity than urease of other species tested, and therefore, it serves as the basis for detection of the organism in gastric biopsies (Hazell *et al.*, 1987). Damage to gastric mucosa induced by urease and ammonia may also provide the organism with the necessary [illegible] Alternatively, urease activity may facilitate nitrogen assimilation by the organism (Marshall *et al.*, 1990). [illegible] demonstrated that [illegible] characteristics compared to those of other bacterial species. First, the enzyme is found in the cytoplasm as well as on the bacterial surfaces, and it displays higher substrate affinity than other bacterial urease (Clayton *et al.*, 1990). Third, it is composed of only two subunits, Ure A and Ure B, whereas other bacterial urease contain three subunits (Hu *et al.*, 1992).

Surface associated urease decreases *H.pylori* survival at neutral pH, low environmental pH reduces urease activity as well as synthesis of nascent urease, catalase and presumably many other proteins. This suggests that *H.pylori* is not acidophilic, although it tolerates short - term exposure to low pH. The bacterium is most probably a neutrophile that has adapted itself to the acidic environment of the stomach and can be classified as an acid - tolerant neutrophile (Marais *et al.*, 1999).

C- Adherence Factor:

Adherence to the gastric mucosa may play an important role in the colonization and pathogencity of *H.pylori.* Approximately one fifth of the organism are adherent to the gastric mucosal surface, whereas the remainder appear to be free - living within the mucus layer (Lee *et al*., 1993). Adherence is species specific and is related to the features of both hosts (e.g. blood group types) and the bacterium (e.g. production of adhesins such as pili and hemagglutinine) (Boren *et al.*, 1993).

Tight attachment of febrillar adhesin on the bacterium to the carbohydrates receptor on the mucosal cell results in the formation of an attaching effacing lesion (adherence pedestal) which it leads actin polymerization and possibly epithelial cell disruption (Tylor et al., 1995). Failure of adherence results in less epithelial cell injury (Peek et al., 1995).

2.5.5.2 Factors Mediating Tissue Injury:

A- Lipopolysacchrides (LPS,s):

Lipopolysacchrides are a family of glycolipids found in the cell envelop of Gram - negative bacteria, including *H.pylori* (Moran, 1996). Lipopolysacchrides, primarily through the lipid A component, stimulate the release of cytokines and posses endotoxic prosperities. Other actions of LPSs include interference with the gastric epithelial cell - laminin interaction, which may lead to loss of mucosal integrity; inhibition of mucin synthesis and stimulation of pepsinogen secretion (Peterson and Graham, 2001).

B- Leukocyte Recruitment and Activating Factors:

H.pylori elaborates a number of lipopolysacchrides independent soluble surface proteins with chemotactic properties to recruit monocyte and neutrophils to lamine propria and to activate these inflammatory cells (Evans *et al.*, 1995). These include *H.pylori* neutrophile - activating protein that expressed by the *nap* A gene and the immunologically active porins (Tufano *et al.*, 1994).

C- Vacuolating Cytotoxin (Vac A):

Approximately (50%) of *H.pylori* strains produce substances that induce vacuole formation in eukaryotic cells. The protein responsible for vacuolation (Vac A) has been purified and the gene encoding the toxin *vac* A has been cloned (Pl...

molecular weight protein that is processed to a mature toxin of 90 K.D. All strains of *H.pylori* possess the *H.pylori vac A* gene but only about (50%) express vacuolating toxin. ...ed ... have demonstrated that supernatants from *H.pylori* strains expressing the toxin, but not those without, cause sever acute superficial mucosal injury (Telford *et al.*, 1994).

D- Cytoxin - Associated Antigen (Cag A):

Cytoxin - associated antigen, a 120 - 140 KD molecular weight highly antigenic protein, is encoded by the *cag* A gene that is part of the cag pathogenicity island (Atherton, 2000). Presence of the cag pathogenicity island is associated with a more prominent inflammatory tissue response than is seen with strains lacking this virulence factor (Atherton *et al.*, 1995).

H.pylori without the cag pathogenicity island have been isolated from patients with peptic ulcer and with gastric cancer showing that increasing risk is not the same as being able to predict outcome (Peterson and Graham, 2001).

E- Outer Membrane Inflammatory Protein (Oip A):

Outer membrane inflammatory proteins a (34KD) outer membrane protein that along with the cag pathogenicity island is associated with an enhanced inflammatory response in the mucosa (Yamaoka *et al*., 2000). Presence of the cag pathogenicity island and Oip A together leads to a more marker inflammatory response than does each one alone. The molecular mechanism of this interaction is not yet known.

F- Heat Shock Protein:

H.pylori expresses two heat shock proteins (Hsp A and Hsp B). They are highly antigenic, but their role in pathogenicity of infection remains unknown. Hsp A binds nickel ions and is a chaperonin (Tomb *et al*., 1997).

characterized by neutrophile infiltration of lamina propria, with time, mononuclear cells, mainly plasma cells and eosinophils enter the lamina properia and eventually outnumber the neutrophils (active chronic gastritis) (Valle *et al.*, 1996). The acute phase lasts (1 to 4) weeks and is replaced gradually by a chronic, mononuclear infiltrate in the lamina propria. Active gastritis refers to the presence of neutrophils mixed with mononuclear cells in the gastric mucosa. Active chronic gastritis occurs in the majority of infected individuals and consists of surface epithelial degeneration, persistent neutrophil infiltration of the epithelium and lamina propria, and mononuclear infiltration (lymphocytes and plasma cells) of the lamina propria which may further induce atrophy and intestinal metaplasia (Arista *et al*., 2001).

Long - term infection by *H.pylori* results in chronic gastritis, a condition manifestation as multiple pathologic entities (Figure 2-2). Persistence of inflammation could, in some individual, determine the development of peptic ulcer (gastric and duodenal) and in others, less frequently MALT (Mucosa Associated Lymphoid Tissue, Lymphoma) (Versalovic, 2003).

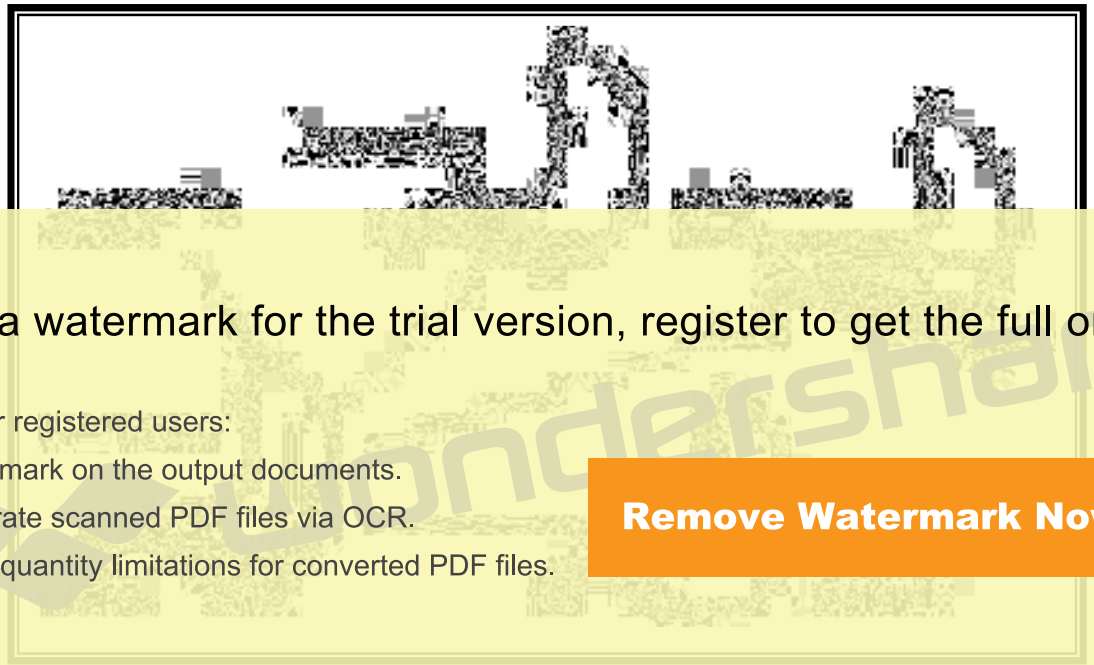


Figure (2-2): Pathogenesis of *H.pylori* - associated gastroduodenal disease, MALT, Mucosa - associated lymphoid tissue (Versalovic, 2003).

In some individuals, chronic *H.pylori* gastritis progresses over time to atrophic gastritis characterized by variable grades of gland loss, which is often associated with intestinal metaplasia (Siurala *et al*., 1987; Versalovic, 2003). These conditions represent intermediate steps in the development of gastric cancer and gastric adenocarcinoma (Correa, 1992).

Bacterial and host factors seem to influence the type of gastro - duodenal disease that is generated. As many as (16%) of *H.pylori* - infected individuals in the United States develop duodenal ulcers in addition to chronic active gastritis. An inverse relationship or paradox exist between the incidence of duodenal ulcer disease and gastric adenocarcinoma (Parsonnet, 1996). Patients with duodenal ulcers rarely develop gastric adenocarcinoma, whereas (3.4%) of patients with gastric ulcers developed gastric adenocarcinoma (Uemura *et al*., 2001). Patients with antral - predominant gastritis are at increased risk for duodenal ulcer disease. In contrast, multifocal atrophic gastric ulcer and gastric adenocarcinoma are not associated with duodenal ulcer disease (Versalovic, 2003).

Seroepidemologic studies indicate that infected individuals if left untreated, a subset of gastric MALT lymphoma. gastric lymphoma MALT lymphomas) that are refractory to therapy (Parsonnet, 1996).

2.5.7 Diagnosis of *H.pylori*:-

Diagnostic tests for *H.pylori* may be divided into those do and those do not require samples of gastric mucosa. Numerous tests are available to detect the presence of *H.pylori* infection. As demonstrated by (Table 2-3), the diagnosis tests for *H.pylori* are categorized as either direct (non invasive) or indirect (invasive) tests (Hopkins and Morris, 1994). No single technique is perfect for the diagnosis of *H.pylori*, therefore, a combination of different test must be performed to obtain the best results.

2.5.7.1 Non - Invasive Tests:

A- Serological Identification of *H.pylori*:

Serological tests mostly based on the ELISA principle detect antibodies to *H.pylori* or its products and are used routinely to screen patients with dyspepsia (Rathbone *et al*., 1985).

Commercially available ELISA kits based on antibodies in sera. It is useful to screen the patients for *H.pylori* infection, usually to find out previous *H.pylori* infection in the community. It is a relatively sensitive and specific test and also inexpensive, and it has limited role in diagnosing acute infection to confirm eradication (Scheiman and Culter, 1999).

Table (2-3): Diagnostic tests for *H.pylori* infection (Hopkins and Morris, 1994)

Application	Test	Method	Comment
Invasive	Rapid urease test	Indirect by urease production	Diagnostic test of choice when endoscope is done.
	Histology	Indirect by morophological features	Evaluates degree of inflammation in gastric tissue.
	Culture	Direct by biochemical properties	Used to determine antimicrobial susceptibility of *H.pylori*.
Non - Invasive	Serology	Indirect by immunological methods	Used for initial diagnosis.
	Urea breath test	Indirect by urease production	Preferred test for evaluating *H.pylori* eradication after treatment.

It is less useful for screening children and unreliable for excluding infection in elderly patients, or as a test for in patients who have received treatment (owing to variable persistence of antibody) (Megraud, 1997).

B- Urea Breath Test:

This test detects bacterial urease activity in the stomach by measuring the output of CO_2 resulting from the splitting of urea into CO_2 and ammonia. A capsule of urea labeled with an isotope of (carbon -14 or -13) is fed to patient. Patient infected with *H.pylori* gives high reading of isotope. The test has excellent sensitivity and specificity but there are drawbacks, carbon -14 is radioactive, albeit weakly, so it is not used in children. Carbon -13 is not radioactive but a mass spectrometer is needed which means that samples must be sent to a specialist laboratory with its attendant (Bazzoli and Pilotto, 1996).

C- Fecal Antigen Test:

This is a promising new test in which polyclonal antibodies are used to detect *H.pylori* antigens in faeces (Vaira *et al*., 1999).

D- Polymerase Chain Reaction (PCR):

Various DNA probes have been developed for the direct detection of *H.pylori* by PCR in gastric juice, faeces, dental plaque and water supplies. Some of them have advantage in the sense they can detect genes expressing antimicrobial resistance and possession of Cag A pathogencity island. Their main drawback is that they are complex to perform and require stringent conditions so they are unsuitable for general use (Peterson, 2000).

2.5.7.2 Invasive Tests:

A- Collection of Specimens:

Mucosal biopsy specimens are taken from the gastric antrum and preferably also from the body of the stomach. For maximum sensitivity, duplicates specimens are taken: one lot for histology (placed in fixative), the other one lot for culture (placed in plain bottles made humid by adding a tiny amount of normal saline). Specimens for culture must be processed as soon as possible within less than 4 hours (McNulty *et al*., 1999).

B- Biopsy and Histology:

Following fixation in formalin, routine Hematoxylin and Eosin (H and E) staining and special stains (e.g. Giems'a stain) are performed for histopathology and organism detection. With Hematoxylin and Eosin [illegible] most *H.pylori* infected individuals with duodenal ulcer or gastric ulcer is found, biopsy specimens are taken from the region surrounding ulcer in the stomach [illegible] found in association with gastric ulcers. Patients with atrophic gastritis often lack prominent inflammation and the sensitivity of histology for organism detection diminishes with increasing severity of glandular atrophy (Versalovic, 2003).

C- Smear Evaluation:

After biopsy specimens are collected with forceps, imprints are made by pressing a needle against the tissue on a glass slide or by simply rubbing the tissue over slide. Cytological specimens may be prepared immediately after biopsy by staining the imprints with rapid Giemsa or Gram stain and *H.pylori* organism may be directly visualized (Parsonnet *et al*., 1988). When imprint smears were used, 30 of 32 biopsy specimens with positive cultures yielded visible organisms by Gram stain (Mirsa *et al*., 1993).

D- Urease Test:

Biopsy urease test is a simple and cheap alternative that can be performed at the bedside. *H.pylori* produced such abundant urease that its action can be detected in biopsy specimens. A specimen is placed into a small quantity of urea solution with an indicator that detects alkalinity resulting from the formation of ammonia by urease. Most infected patients (70%) give a positive result within (2hr) (90%) after (24hr) (Hazell *et al*., 1987; Marshall *et al*., 1987). The sensitivity of rapid urease testing in maximized if specimens are obtained from gastric angle and multiple specimens are obtained (Megraud, 1997).

E- Culture and Isolation:

increase prevalence of drug resistant *H.pylori* [illegible] necessitate susceptibility testing when the frequency of resistance to [illegible] increases [illegible] those cases [illegible] therapy (Gra[illegible]) a fastidious organism that requires an enriched transport medium if the biopsy specimen is not plated for culture within (2hr). Because optimal transport media are not available commercially and microscopic plating is usually not performed in the endoscopy unit, routine cultures have not acquired general acceptance in the United States. *Helicobacter* typically grows best in freshly prepared, moist media incubated in a warm (37°C) temperature, and an atmosphere of (5 to 10)% carbon dioxide, (80 to 90)% nitrogen and (5 to 10)% oxygen. A humid atmosphere enriched in hydrogen content (5 to 8)% improves the yield of *H.pylori* (Murray *et al.*, 1999). Primary isolation of *H.pylori* from gastric biopsy specimens requires (5 to 7) days under a microaerophilic conditions.

Selective media (on plates) enriched with blood or serum are recommended, such a strategy maximizes the sensitivity of culture that used for cultivation of *H.pylori.* Ideally, tissue specimens should be processed as quickly as possible in order to increase the chance of isolating *H.pylori*. In the case of delay, it should be stored at (4°C) and cultured within (5hr) (Mergaud *et al*., 1997). The fragility of bacteria can lead to a negative result if transport conditions are not carefully followed up (Megraud *et al*., 1997) specimens are either minced (Goodwin *et al.*, 1985) or ground with a glass grinder before inoculation. Grinding the biopsy specimens gave much heavier growth of *H.pylori* than merely mincing them (Collee *et al*., 1996).

Numerous types of culture media used to isolate *H.pylori* are suggested. A blood based media have been widely used whether chocolate or blood agar with or without supplementation of antibiotics (Hachem *et al*., 1995).

recommended .Vancomycin, inhibits Gram positive from the oropharynx. Cyclohexmide is used as antifungal, and trimethoprim, polymyxin B, nalidix acid and celsulodin to inhibit Gram negative contaminants such as *Pseudomonas aerogenosa* which is a common contaminant of gastric biopsy specimens. Amphotercin B is used to inhibit yeast that often contaminant specimens from the stomach (Dent and McNulty, 1988).

Plates inoculated with the gastric biopsy should be incubated under microaerophilic conditions for 1 week at (35 – 37)°C (Goodwin *et al.*, 1985). The identity of any isolates is confirmed by Gram stain from one of the colonize and testing for the presence of catalase, oxidase

and urease (Baron *et al.*, 1994; Quiroz *et al.*, 1999). *H.pylori* exhibits patchy distribution in gastric mucosa, hence, multiple biopsies should be taken from different sites in the gastric antrum and the body (Hopkins and Morris, 1994).

Failure to grow *H.pylori* from appropriate sample may be due to chemical agents used during endoscopy which act as antibacterial to *H.pylori*. Benzocaine is inhibitory to *H.pylori* but lidocaine is not. Antibiotics bismuth preparation, inadequate specimens or failure in the microbiological technique all can lead to failure in culturing *H.pylori* (Blaser *et al.*, 1997).

2.5.8 Treatment of *H.pylori*

In the decade of 1980, treatment for *H.pylori* was difficult since combinations of bismuth, tetracycline, metronidazole were acquired for eradication of the organism (Bor[illegible]). A breakthrough came when Unge and colleagues, 1989, in Sweden observed that the action of amoxicillin was greatly enhanced when gastric acid was suppressed with a proton pump inhibitor, notably omeprazole. Thus, since 1996, *H.pylori* has been able to be treated relatively easily with a 7 days therapy of omperazole (to render the gastric pH neutral) in combination with two antibiotics, usually amoxicillin and clarithromycin. Omeprazole, clarthromycin and metronidazole combination have achieved similar high cure rates, notably in Italy (Bazzoli, 1999). For the difficulty to eradicate infections, bismuth, tetracycline, metronidazole and omeprazole are usually successful. These therapies have proven that peptic ulcer is mostly a bacterial infection unrelated to the victims emotional state (Kung *et al.*, 1997).

However, antimicrobial therapy has a number of inherent limitations that might be overcome by use of an effective vaccine or combined regimen of antibiotics and vaccine. Primary treatment failure occurs in (15%) of patients treated with antibiotics combined with antisecrectory drug. Poor compliance with antibiotic regimens and antibiotic resistance in *H.pylori* (Noach *et al.*, 1994) contribute to treatment failures.

Several reasons have been proposed to explain the clinical failure after treatment, insufficient concentration of active drugs in gastric mucus, instability of some agents at an acidic pH inappropriate formulation of drug, insufficient duration of treatment and variable compliance of patients. Recently, it has rapidly acquired resistance to some antibiotics and this event might also account for clinical failure. Also misuse of antibiotics that would favour selection of antibiotic resistant *H.pylori* and negatively affect the ecology of gastric microflora (Kobayaski *et al.*, 2004).

Recurrent after successful treatment is associated with slow improvement in submucosal inflammation. One year after successful treatment, neutrophil infiltration resolves; lymphocyte infiltration and lymphoid follicles improve but persist (Genta *et al.*, 1994).

The National Institutes of Health recommends treating only persons with active ulcer disease (gastric or duodenal) or persons receiving maintenance therapy for recurrent ulcer disease (NIH, 1994). Hack *et al.*, (1994) reported that treatment could be cost - effective approach to preventing ulcer disease, dyspepsia and cancer in populations of high risk. Graham, (1994) mentions that eradicating infection in all infected persons, regardless of symptoms, is the correct approach. Consequently, it is likely that diagnosis and therapy will be offered to a wider segment of the population to prevent adverse outcomes of infection.

2.5.9 Probiotic and *H.pylori* Eradication:-

A probiotic is defined as "a live microbial food ingredient that is beneficial to health". Probiotic bacteria are used to treat distributed intestinal microflora, it may help in vaginal bacterial infection, urinary tract, lactose intolerance, diarrhea and colon cancer. They are usually measured in numbers of organisms per gram supplements, typically contain four billion or more organisms per gram. Probiotic have the ability to survive passage through the gastrointestinal tract and are usually considered to be non-pathogenic (Hoolihan, 2001). Researches supported a beneficial effect of probiotic consumption include: improving intestinal tract health, enhancing the bioavailability of nutrients, reducing symptoms of lactose intolerance, decreasing the prevalence of allergy in susceptible individual and reduction risk of certain cancers (Kabir *et al*., 1997).

The mechanisms by which probiotics exert their effects are largely unknown but may involve modifying gut pH, antagonizing pathogens through the secretion of antimicrobial [illegible] competing for pathogen binding and receptor sites, enhancing the availability of nutrients and growth factors, stimulating immunodulatory cells and producing lactose (Isolauri *et al*., 1991 ; Noback *et al*., 2000). It was reported that *Lactobacilli* can inhibit the growth of *H.pylori* in vitro and exhibits antagonistic activity against it. (Bhatia *et al.*, 1989; Kabir *et al.*, 1997) .In human volunteers, the spent culture supernatant of human *Lactobacillus acidophilus* strain LA1 is active against *H.pylori* (Michette *et al.*, 1995).

The need for new strategies for *H.pylori* eradication, alternative or complementary to antibiotic therapy, has recently claimed the attention of many investigators. Pre - clinical studies have shown the inhibition of *H.pylori* growth by *Lactobacilli* and anti - *H.pylori* action of *Lactobacillus salivarius*, *Lactobacillus acidophilus* and *Lactobacillus casei*; subspecies *rhamnosus* strains, possibly due to the production of lactic acid or to the secretion of autolysin (Midolo *et al*., 1995).

Clinical studies have demonstrated a persistent reduction in delta over baseline values at the carbon 13 of urea breath test in dependently of omperazole administration with *Lactobacillus acidophilus* La1. The eradication was in 6 out of 14 patients with *Lactobacillus acidophilus* alone. Positive results in patients in which a standard *H.pylori* triple therapy was randomly supplemented with *L.acidophilus* (Canduccif *et al.*, 2000). Also there is some preliminary evidence that probiotic bacteria may inhibit the gastric colonization and activity to *H.pylori*, which is associated with gastritis, peptic ulcer and gastric cancer. *L.salivarius* was found to inhibit *H.pylori* colonization in vitro studies as well as in mice (Kabir *et al.*, 1997; Aiba *et al.*, 1998). Inhibition of *H.pylori* infection was also shown in humans consuming *L.johnsonii* (Michetti *et al.*, 1999).

L.acidophilus could be effective in increasing eradication rates of standard anti *H.pylori* therapy (Noback *et al.*, 2000).

Probiotic are also effective in reducing the side effects of "triple therapy" with antbiotic used to eradicate *H.pylori* from the stomach. *L.rhamnosus* CG reduces the incidence of diarrhea, nausea and taste disturbance and tinidazole receiving rabeprazole, clarithromycin and tinidazole for *H.pylori* eradication (Kieran *et al.*, 2003).

Chapter Three

Materials and Methods

3. Materials and Methods

3.1 Materials:

3.1.1 Apparatus:-

Apparatus	Company (Origin)
Autoclave	Gallenkamp (England)
Centrifuge	Gallenkamp
Cooling Centrifuge	Sigma (U.S.A)
Distillater	Gallenkamp
Electrical sensitive balance	Mettler (Switzerland)
Electrical oven	Gallenkamp
Haemocytometer	Neubauer (Germany)
Handwork homogenizer	Virtis (U.S.A)
Incubator	Gallenkamp
Compound light microscope	Olympus
Microtome	Leitz (Germany)
Orbital incubator	Gallenkamp
Paraffin dispenser	Lipshow
pH meter	Orient research (U.S.A)
Sonicator	MSE (England)
Spectrophotometer	Hitachi (Japan)
Tissue processor	Shandon Southern (England)
Vortex mixer	Labeco (Germany)
Waterbath	Gallenkamp

3.1.2 Equipment:-

Equipment	Company (Origin)
Anaerobic Jar	Oxoid (England)
Disposable syringes (2ml and 5ml)	BROMED (U.S.A)
Filter papers (0.22)&(0.44)µm	Millipore (U.S.A)
Gas generating kit (gas Pak)	Oxoid
Gas generating kit (gas Pak)	Al-Razi (Iraq)
Glass petridishes	BROMED (Germany)
Microlitter pipettes	Brand (Germany)
Millipore unit	Gallenkamp (England)
Multi well plats scalpel	Boihit (Finland)
Plain tubes (10ml)	BROMED (U.S.A)

3.1.3 Ready to Use Media (Powders):

Medium	Company (Origin)
Brain - heart infusion agar	Biolife (Italy)
Brain - heart infusion broth	Oxiod (England)
Brucella agar medium	Oxiod
Blood base agar medium	Oxiod
Columbia base agar medium	Biolife
Muller Hinton agar medium	Biolife
Modified Regoza agar	Biolife
Urea agar base	Oxiod
Urea broth	Oxiod

3.1.3.2 Laboratory Prepared Media:

The following media were prepared in the laboratory: Blood agar, chocolate agar, selective brucella, Columbia, Brain - heart infusion agar and Brain - heart infusion broth (BHI-VAN). Modified Regoza broth, motility medium and urea base agar.

3.1.4 Chemicals:-

Chemical	Company (Origin)
Acetic acid	BDH (England)
Ammonium chloride	BDH
Acetone	BDH
Benedict's	Gurr (England)
Chloroform	BDH
Crystal violet	BDH
Dilute carbol fucshin	BDH
Ethanol	BDH
Eosin	BDH
EDTA	LTD (England)
Formalin	BDH
Ferrus sulfate	BDH
Giemsa	BDH
Glycerol	BDH

Chemical	Company (Origin)
Heparin	Leo pharma ceutical products (England)
Hydrochloric acid	BDH
Hydrogen peroxide (H2O2)	BDH
Methyl alcohol	Merek (Germany)
Magnesium sulfate	Fluka (Germany)
Manganese sulfate	Fluka
Propyl alcohol	Merek
Potassium chloride	Fluka
Sodium chloride	Sigma (U.S.A.)
Sodium pyruvate	Oxiod
Sodium acetate trihydrate	BDH
Sodium citrate	Fluka
Sodium hydroxide	Fluka
Sucrose	Sigma
Triammonium citrate	Fluka
Tween 80	Sigma
Urea powder	Oxiod

3.1.5 Antibiotics:-

Two forms of antibiotics were used in this study, antibiotic disks and antibiotic powders.

3.1.5.1 Antibiotic Disks:

Antibiotic	Symbol	Conc. (µg/disk)	Company (Origin)
Amikacin	AK	30	Oxoid (England)
Amoxicillin	AMX	20	Oxoid
Ampicillin	AMP	10	Al-Razi Co.
Ceftriaxone	CRO	30	Al-Razi Co.
Cephalothin	KF	30	Oxoid
Ciprofloxacin	CIP	5	Al-Razi Co.
Clarithromycin	Crr	15	Oxoid
Erythromycin	E	30	Oxoid
Metronidazole	M_t	5	Oxoid
Nalidixic acid	NA	30	Oxoid
Penicillin G	PG	10	Al-Razi Co.
Tetracycline	TE	30	Al-Razi Co.

3.1.5.2 Antibiotic Powders:

Antibiotic	Company (Origin)
Amphotricin B	Squibb and Sonsltd (England)
Amoxicillin	SDI (Iraq)
Clarithromycin	Oxoid (England)
Metronidazole	SDI
Nalidixic acid	SDI
Pencillin G	SDI
Polymyxin	Oxoid
Trimethoprim	SDI
Tetracycline	SDI
Vancomycin	Qualigand (France)

(NCCLS, 1991)

Kit	Company (Origin)
Enzyme Linked Immuno Sorbent Assay (ELISA).	Biohit (Finland).

* Reagents preparation and materials used were provided by manufacturing company (Appendix I).

3.1.7 Bacterial Strain:-

Probiotic Isolates	Supplied by
Lactobacillus acidophilus.	Biotechnology Department/College of Science/Al- Nahrain University.

3.1.8 Buffers, Solutions, Reagents and Stains:-

- Phosphate Buffer Saline.
- Formal Saline Buffer.
- Physiological Saline Solution.
- Antibiotic Solution.
- Fixative Solution.
- Sodium Bicarbonate Solution.
- Staining Solution.
- Giemsa Stain Solution.
- Hematoxyline - Eosin Stain Solution.
- Catalase Reagent.
- Oxidase Reagent.

3.2 Methods:

3.2.1 Media Preparation:

Ready to Use Medium:

The media listed in (3.1.3.1) were prepared according to instructions of the manufacturer. They were brought to boil in water to dissolve all constituents completely, then the pH was adjusted to 7.2 and sterilized by autoclaving at (121°C) (15Ib/In^2) for (15min). They were incubated at (37°C) for (24hr) to ensure sterilization. Antibiotics used in the medium were previously sterilized by filtration using (0.22µm) Millipore filter.

3.2.1.2 Laboratory Prepared Media:

A- Brain Heart Infusion Broth (Transport Medium):-

It was prepared according to manufacturer information fixed on the container. After sterilization, aliquot of (5ml) was dispensed into (10ml) sterilized test tube (Megraud *et al*., 1997).

B- Selective Brain Heart Agar Base:-

A quantity of (47g) of Brain - heart infusion agar was dissolved in (90ml) distilled water and sterilized. After cooling to (45-50)°C, (7%) of horse or human blood was added.

Vancomycin, trimethoprim, amphotricin B and polymyxin (10, 5, 10 and 5)µg/ml respectively were added to make the medium selective for primary isolation (Ansorg *et al*., 1991).

C- Brucella Agar Medium:-

It was prepared according to manufacturer information fixed on the container, after sterilization cooled a (7%) horse or human blood was added. The medium was supplemented with vancomycin, trimethoprim, amphotricin B and polymyxin (10, 5, 10 and 5)µg/ml respectively as a selective medium for *Helicobacter pylori* isolation (Peiccolomini *et al*., 1990).

D- Blood Agar Medium:-

It was prepared by adding (5%) horse or human blood to the previously autoclaved blood agar base. After mixing, the medium was poured into petridishes. This medium was used for the isolation and identification of *H.pylori* (Atlas *et al*., 1995).

E- Chocolate Agar (Brain - heart Infusion Agar Base):-

This media was prepared as described by Hachem *et al*., (1995), by adding (7%) horse or human blood to the Brain - heart infusion agar then heating the medium until it turned to brown color. Then the medium cooled to (45-50)°C. This medium was used for primary isolation of *Helicobacter* and in subculturing in secondary isolation.

F- Columbia Agar Medium:-

This medium was prepared according to the manufacturers instructions fixed on the container. After sterilization, the medium was cooled to (45-50)°C, then (7%) horse or human blood was added. Vancomycin, trimethoprim, amphotricin B and polymyxin (10, 5, 10 and 5)µg/ml were added to make medium selective for primary isolation (Hazell, 1993).

G- Brain Heart Infusion - Vancomycin Amphotricin Nalidixic Acid Broth (BHI - VAN):-

This medium was prepared as described by Siu *et al.*, (1998) by adding (5%) horse serum, (0.25%) yeast extract, (6µg/ml) vancomycin, (4µg/ml) amphotricim B and (20µg/ml) nalidixic acid into previously sterile Brain heart infusion broth (BHI - VAN), aliquot of (5ml) was dispensed into sterile test tube. This medium was used for preservation of [illegible] specimens during transportation.

[illegible] Broth Medium (MRS)

It was prepared according to DeMan (1960) by dissolving the following ingredients in 950 milliliter of distilled water; peptone (10g), meat extract (10g), yeast extract (5g), glucose (20g), tween 80 (1ml), K2HPO4 (2g), sodium acetate hydrate (5g), triammonium citrate (2g), $MgSO_4$. $7H_2O$ (0.2g), $MnSO_4$. $4H_2O$ (0.05g). pH was adjusted to 6.0, the medium then autoclaved. This medium was used for growing lactic acid bacteria (*Lactobacillus acidophilus*).

I- Muller – Hinton Agar Medium (Chaves *et al.*, 1999):-

This medium was prepared according to the manufacturer instructions. After sterilization and cooling to (45-50)°C, (7%) horse or human blood was added to medium and dispensed into petridishes. This medium was used for antibiotic susceptibility test and MIC determination test.

J- Motility Medium:-

It was prepared according to the Cruikshank *et al.*, (1975) by dissolving (3.7g) Brain - heart infusion broth (instead of nutrient broth) and (4g) agar - agar in 950 milliliter of distilled water. pH was adjusted to 7 then the medium was autoclaved. This medium was used for motility test.

K- Urea Agar Base Medium:-

Urea agar medium was prepared by adding (5ml) of sterile (40%) of urea solution to (95ml) of a cool sterile urea agar base at (50°C). the medium mixed well, (5ml) aliquots were dispensed into sterile test tubes and left to solidify as slants, then kept tightly closed in a cool dry place (Marshall *et al.*, 1984).

3.2.2 Preparation of Buffers, Solutions, Reagents and Atoms:-

3.2.2.1 Buffers and Solutions

Phosphate buffer [pH = 7.0

It was prepared by dissolving (0.85g) of sodium chloride in 950 milliliter of distilled water, sterilized by autoclaving (Manniatis *et al.*, 1982).

B- Formal Saline Buffer:-

Ten milliliters of formalin (4%) was dissolved in (90ml) of saline solution to preserve gastric biopsy specimens for histological investigation (Murry *et al.*, 1999).

C- Phosphate Buffer Saline (PBS):-

It was prepared according to Gruikshank *et al.*, (1975) as follows: Dissolving (8g) NaCl, (0.2g) KCl, (0.2g) KH_2PO_4 and (1.15g) Na_2HPO_4 into 950 milliliter distilled water, sterilized by autoclaving and used for preservation of uroepithelium and bacteria cells.

D- Fixative Solution:-

It was prepared by mixing (30ml) of methanol with (10ml) of acetic acid. It was used for fixation of bacteria and uroepithelial cells during staining by methylene blue (Atlas *et al.*, 1995).

E- Sodium Bicarbonate Solution:-

It was prepared by dissolving (5.6g) $NaHCO_3$ in (95ml) distilled water. The solution sterilized by filtration using (0.22µm) pore size Millipore filter then dispensed into (20ml) volume and stored at (4°C). It was used in Giemsa stain preparation (Metacalf *et al.*, 1986).

G- Haematoxyline - Eosin Stain Solution:-

- Haematoxyline stain.
- Aqueous eosin stain: (1g) aqueous eosin added to (99ml) of distilled water.
- Crystal violate stain: (1g) crystal violate powder dissolved with (80ml) of distilled water and (20ml) of ethanol (95%).
- Logal iodine: (1g) iodine and (2g) (KI2) dissolved with (300ml) distilled water.
- Sodium bicarbonate solution: (0.5mg) sodium bicarbonate dissolved with (100ml) of tap water.

- Acid alcohol: three drops of concentrated HCl dissolved with (200ml) ethanol (50%).
- Giemsa stain solution.
- Acetic acid solution (0.5 %).
- Xylol.

L- Antibiotic Solutions:-

All antibiotic solutions (except tetracycline which dissolves in ethanol) were prepared as described by Manniatis *et al.*, (1982). This was carried out by dissolving (0.1g) of antibiotic with (10ml) sterile water, then sterilized by filtration through Millipore filters (0.22µm) and stored at (4°C).

B- Oxidase Reagent:-

A solution of (1%) N, N, NN - tetramethyl - para - phnylene diamine dihydro - chloride was prepared in sterile distilled water then kept in dark.

3.2.3 Sterilization:-

Three methods of sterilization were used:

3.2.3.1 Moist - Heat Sterilization:

All media and solutions were sterilized by autoclaving at (121°C) (15Ib/In^2) for (15min).

3.2.3.2 Dry - Heat Sterilization:

Electric oven was used to sterilize glassware and petridishes at (160-180)°C for (2 – 3)hr

3.2.3.3 Membrane Sterilization (Ultrafiltration):

Millipore filters (0.22µm) were used to sterilize antibiotic solutions and the growth filtrates of *Lactobacillus acidophilus*.

3.2.4 Sampling:-

3.2.4.1 Study Subjects:

A total of 300 samples were collected from sheep milk, sheep, bovine and human gastric tissues during the period from 25/4/2014 to 7/9/2015. They were distributed as follows:

- One hundred sample of sheep gastric tissues.
- Fifty samples of sheep milk.
- Twenty samples of bovine gastric tissues.
- One hundred and thirty samples of patients suffering from upper gastrointestinal complain.
- Fifteen samples of blood obtained from normal persons used as control in ELISA test.

A- Patient Groups:-

This included 130 patients with various gastrointestinal symptoms representing different age groups from both sexes. Samples were obtained from Endoscopy Department of Gastroenterology and Hepatology Teaching hospital and Baghdad Medical City in Baghdad. Informed written consent was obtained in advance from each patient.

B- Animal Groups:-

This included 170 samples from different animals of Sheikh Maroof slaughter house and Alwa' Al-Doora (Al-Kurkh) in Baghdad.

3.2.5 Samples Collection and Treatment:-

3.2.5.1 Animal Biopsy Specimens:

Eight gastric tissue sections of (3-4)cm were obtained from each of the ruminant, abomasums, omasum and reticulum regions of sheep using sterile forceps (Dore 1999). One tissue section from each region was fixed in (10%) formal buffer saline for histological investigation and the other one was used for bacteriological investigation. The sections were transported to the laboratory in (0.5ml) BHI - VAN broth with ice and kept for no longer than (4hr) before analysis. The same procedure was repeated for obtaining gastric tissue samples.

Fifty sheep samples were collected [illegible] dilutions were made from last dilution (Brain - heart infusion, Columbia and Brucella agar) plates after pouring each of Brain - heart infusion, Columbia and Brucella agar plates were incubated at (37°C) under microaerophilic conditions in an anaerobic jar with a gas generating kit. Plates were examined for positive results at interval of (3, 5, 10 and 14) days before discarded as negative.

3.2.5.2 Human Biopsy Specimens:

Patients were advised to fast for overnight before endoscopy. Endoscopies performed under local anesthesia (xylocaine) (Querioz *et al.*, 1987). The endoscopy was disinfected with (2%) glutaraldehyde (cidex) before and after each procedure (Megraud *et al.* 1985; Simor *et al.*, 1990). Biopsy forceps were washed with water and disinfected with

glutaraldehyde (cidex) for (10min), then washed with distilled water before each procedure. During upper gastrointestinal endoscopy, four gastric biopsy specimens were taken (3-4)cm, two from each of corpus (body) and antrum region of the stomach (Hudson *et al.*, 1993). Standard pinch biopsy forceps were used. One biopsy from each region was fixed in (10%) formal buffer saline for histological investigation and the other was used for bacteriological investigation. Biopsy specimens were transported to the laboratory in (0.5ml) Brain - heart infusion broth with ice and kept at (4°C) for no longer than (4hr) before processing.

3.2.5.3 Blood Samples:

Five milliliters of venous blood were taken in dry - sterile tube from 75 patients for serological test. After clotting, the sera were separated by centrifugation for (10min) at (3000rpm), then divided into aliquots and stored at -20°C until use.

3.2.6 Laboratory Treatment:-

The biopsy samples were minced and homogenized between the frosted ends of sterile microscope sides in a sterile petridishes near benzene burner, then subjected for the following tests:

3.2.6.1 Biopsy Urease Test:

The first minced biopsy sample was inoculated on urea agar slant containing phenol red (as an indicator) and incubated at (37°C). Slants were examined for color change from yellow to pink before and after (1hr) and after (24hr). The test was not finally declared as negative till (24hr) (Hazell *et al*., 1987; Megraud, 1997).

3.2.6.2 Direct Biopsy Smear (Glupczynski *et al.*, 1988):

A liquot of a biopsy sample was smeared on a glass slide to be stained with a rapid Giemsa or Gram stain and *H.pylori* was directly be visualized.

3.2.6.3 Biopsy Culturing:

The second minced biopsy was inoculated in Brain - heart infusion broth and on each of selective (Columbia, Brain - heart infusion and Brucella) agar media plates and non selective (Chocolate and Blood) agar media plates that was used for primary isolation of *H.pylori*. The cultures were incubated at (37°C) under microaerophilic conditions in an anaerobic jar with a gas generating kit. Plates were examined for positive growth for intervals of (3-5) days for the selective media and 7 days for the nonselective media before discarding as negative. For positive growth, the colonies must be tiny, clustering, translu [illegible]

3.2.7 Identification of *Helicobacter pylori*:-

3.2.7.1 Gram Staining:

Dry heat fixed smears were taken from colonies and placed on microscopic glass slide to examine the morphology of bacteria.

3.2.7.2 Biochemical tests of *H.pylori*:

A- Urease Test of Colonies:-

Grown colonies were picked from the agar plate with a sterile loop and then inoculated on urea agar slant. Positive result was detected by changing color from yellow to pink within few minutes.

B- Catalase Test:-

One drop of hydrogen peroxide (3%) was added to part of the grown isolated colonies which were picked up from the agar with a woody stick on the surface of a sterile slide. Production of gas bubbles within (20-30) seconds from *H.pylori* growth on the slide indicated a positive reaction.

C- Oxidase Test:-

A few drops of freshly made oxidase reagent (1%) were added on a strip of filter paper and then an isolated colony was rubbed by using a strike woody sticks. A positive reaction is indicated by an intense deep purple color appearing within (5-10) seconds.

3.2.7.4 Growth at (25 °C) and (45 °C):

Plates of Brain - heart infusion agar were inoculated with *H.pylori*, then incubated at (25°C) and (45°C) for (24-48) hr. Positive results were obtained by the appearance of *H.pylori* growth.

3.2.7.5 Susceptibility Test for Nalidixic Acid and Cephalothin:

Isolates were inoculated in sterile brain-heart infusion broth then incubated at (37°C) for (24-48) hr then, (0.1ml) of this inoculum was spreaded on Muller-Hinton agar supplemented with (5%) horse blood and then with a sterile forceps, cephalothin and nalidixic acid disks were placed on the surface of inoculated plate and incubated at (37°C) for (24hr) under microaerophilic conditions.

3.2.8 Maintenance of *Helicobacter pylori* Isolates:-

Maintenance of bacterial isolates was performed according to Han *et al*., (1995) as follows:

3.2.8.1 Short - Term Storage:

Isolates of bacteria were maintained for few weeks on Brain - heart infusion agar supplemented with (7%) horse blood. The plates were tightly wrapped with parafilm and then stored at (4°C).

3.2.8.2 Medium - Term Storage:

Isolates of bacteria were maintained as stab cultures for few months. Such cultures were prepared in small screw - capped bottles containing (2-3) ml of chocolate agar medium and stored at (-20°C).

3.2.9 Histological Examinations:-

Histological examination was diagn[illegible] histopathologist. The biopsy specimen which was fixed in (10%) formalin solution was washed by tap water for few minutes and left in ethanol (50%) for (30min) while (70%) ethanol was used to keep the specimen for a long time. The specimen was transferred to (2.5% absolute ethanol + 75% butanol) and left for (2hr). Paraffin wax sectioned in (4µm) thickness to be easier to use, then specimen was stained with Giemsa and hematoxyline - eosin stain.

3.2.9.1 Giemsa Stain Method (Luna, 1968):

- Histological sections were placed in the oven at (70°C) for 15min
- Paraffin was dewaxed by placing the sections in xylol for (5-10) sec. and then dehydrated in graded serial of ethanol (99.8%, 95% and 70%), respectively for (5-10) sec.

- They were put in Giemsa stain with a concentration of (14.2%).
- Placed in (1%) acetic acid for (1-2) min
- Placed in propanol for (1-2) sec.
- Placed in absolute alcohol for one min
- Placed in xylol and then mounted in canada balsam.

3.2.9.2 Hematoxylin - Eosin Method (Guyer, 1953):

Histological sections were placed in the following solutions and reagents as follows:

- Xylol for (5min).
- Absolute alcohol for (1min).
- A graded series of ethanol (80%, 70%, 50% and 30%) for (1min) then rinsed in distilled water for 1min.
- Sodium- hydrosulphate to erase the tissue to white and rinsed in tap water for few min
- Hematoxyline for (1-5) min then rinsed in tap water to get rid of the excess dye.
- Acid alcohol, till the color turned to pink then rinsed in tap water.
- Na- bicarbonate till the color turned to blue, then rinsed in tap water.
- Eosin for 5min, then rinsed in tap water.
- Crystal violet dye for (2min).
- Lugol - iodine dye for (3min) and left to dry for few min
- Xylol for (3min), then mounted in Canada balsam.

3.2.10 Serological Tests:-

3.2.10.1 Enzyme Linked Immuno Sorbant Assay (ELISA):

Bioelisa *Helicobacter* IgG is an ELISA test for quantitative and qualitative of IgG antibodies specific to *H.pylori* in human serum. It's based on an enzyme immuno assay technique with partially purified *H.pylori* bacterial antigen absorbed on a microplate and a detection antibody labeled with horse radish peroxidase (HRP). This procedure was conducted as following:

- Before running the assay, the reagents were allowed for few min to reach room temp.
- A liquot of (100µl) of each calibrator which included a diluted human IgG and *H.pylori* standardized at (10, 20, 120 and 640) AU/ml was dispended into wells.
- A liquot of (100µl) of each patient serum was dispended into the corresponding wells and incubated for (45 min) at (37°C).
- Wells were washed times using
- A liquot of (100µl) of the conjugate was added to each well, except for blank and incubated for (45min) at (37°C).
- Wells were washed by repeating step (d).
- A liquot of (100µl) of substrate solution was dispensed in all wells including the blank and incubated for (30min) at (37°C).
- Reaction was stopped by adding (100µl) of stop solution into each well in the same sequence and timing as for substrate addition.
- Samples with an absorbance value equal to or greater than the cut - off value (which is equal to 20AU/ml) were considered to be positive for *H.pylori* IgG antibodies, while those with an absorbance value lower than the cut-off value were considered as negative for *H.pylori* IgG antibodies.
- Readings were performed at (450nm), setting the zero with the substrate blank.

3.2.11 Antibiotic Susceptibility Test (Bauer and Kibry, 1966):-

Five milliters of brain heart infusion broth supplemented with (5%) horse or human sera were inoculated by *H.pylori* isolates, then incubated at (37°C) for (24hr). A liquot of (0.1ml) of the inoculated broth was transferred and spreaded by sterile cotton swab on Muller - Hinton agar plates supplemented with (5%) horse blood in three different planes (by rotating the plate approximately 60° each time to obtain an even distribution of the inoculum). The inoculated plates were then placed at room temperature for (10-15) min to allow absorption of excess moisture. With a sterile forceps, the selected antibiotic disks were placed on the inoculated plates and incubated at (37°C) under microaerophilic conditions for (48hr). After incubation, the diameters of inhibition zones were measured by a ruler (mm). Results were determined and compared according to National Committee for Clinical Laboratory Standards (NCCLs, 1991).

Minimum Inhibitory Determination:-

Minimum inhibitory concentration (MIC) was determined by agar dilution method (NCCLs, 2000). Agar dilutions were prepared using Muller-Hinton agar medium containing (5%) horse blood supplemented with two fold serial dilutions of metronidazole, clarithromycin, amoxicillin, tetracycline and penicillin stock solutions as prepared (3.2.2.1.L) ranging from (16-1024) µg/ml. Fresh *H.pylori* isolates were inoculated in sterile Brain - heart infusion broth then incubated at (37°C) for (24hr) under microaerophilic conditions. Then, (0.1ml) of each inoculum was delivered to the agar dilution plate. All plates were incubated at (37°C) under microaerophilic conditions for (24hr). The MIC was defined as the lowest concentration of antibiotic solution that completely inhibited the growth of the inoculum.

3.2.13 Testing the Inhibitory Activity of Lactic Acid Bacteria (LAB):-

3.2.13.1 on Solid Medium (MRS Agar):

A culture of *Lactobacillus acidophilus* previously grown in MRS broth was streaked on MRS agar, then incubated under anaerobic conditions at (37°C) for 24hr (Silva *et al*., 1987). After incubation, a cork borer (5mm) was used to withdraw discs of *L.acidophilus* growth and put on surface of Brain - heart infusion agar that was previously inoculated overnight with (0.1ml) of *H.pylori* isolate by a spreader, then incubated at (37°C) for (24hr) under microaerophilic conditions.

The same procedure was repeated using different incubation times of LAB (24, 48 and 72) hr to determine the optimum incubation time that gives greater inhibition effect.

3.2.13.2 in Liquid Medium (MRS Br[illegible]

[illegible] (1%) of *L.acidophilus* cult[illegible] at (37°C) for different periods of time (24, 48, 72) hr. After incubation, the culture was centrifuged at 6000 rpm for (15min) and the supernatant was obtained (Aiba *et al*., 1998; Michett *et al*., 1999).

After adjusting pH of the filtrate to 6.5 using (0.4N) NaOH, it was filtered through Millipore filter units (0.22µm), then well diffusion method that mentioned by Vignolo *et al*., (1993) was applied, when 0.1 ml of an overnight *H.pylori* isolates were inoculated by spreader on Brian - heart infusion agar plates, (5mm) well was made by a cork borer. Each well was filled with *L.acidophilus* filtrate and incubated at (37°C) for (24 and 48) hr. Inhibition zones around the well were measured (in mm) and compared with the control which contained MRS broth without the bacteria (Vignolo *et al*., 1993).

The filtrates were concentrated by the freeze - dryer. Well diffusion method was repeated against *H.pylori* isolates. Control was containing concentrated MRS broth without *L.acidophilus.*

Equal volume (100ml) of MRS broth was inoculated with concentrated filtrates of LAB previously prepared by freeze - dryer (lyophilizer) with 1 ml LAB concentrated to one- fold (100ml), two -fold (50ml), three- fold (25ml) and four- fold (12.5ml).

3.2.13.3 Determination of Minimum Inhibitory Concentration of *Lactobacillus acidophilus* Concentrated Filtrates:

Serial dilutions (10ml) of four fold concentrated filtrate of *L.acidophilus* which prepared previously were prepared by using Brain - heart infusion broth for dilution. After the following dilutions; (1:0, 1:1, 2:1, 3:1, 4:1, 6:4, 7:3, 8:2, 9:1, were prepared in test tubes and filtrates in Brain - heart infusion broth. The tubes were with 0.1 ml [illegible] isolates each, then incubated ove[illegible] of each tube was observed and recorded as slight (+), medium (++), heavy (+++) and no growth (-). Growth was estimated by using spectrophotometer and optical density ($O.D_{600}$) was read for each dilution. Results were matched with the growth intensities (Midolo *et al.*, 1995).

3.2.14 Bacterial Adhesion Test (Iwahi *et al.,* 1982):-

3.2.14.1 Preparation of *H.pylori* Suspension:

Ten milliliter of Brain - heart infusion broth medium was inoculated with bacterial growth culture, then incubated at (37°C) under microaerophilic conditions for (24hr), there after, culture of bacteria was washed twice with PBS and concentrated by centrifugation at (1000rpm) for (20min) and resuspended in PBS.

3.2.14.2 Preparation of Epithelial Cells:

Uroepithelial cells were isolated from urine of some healthy females by centrifugation at 1000 rpm for 5min then washed three times with PBS and recentrifuged at 1000 rpm for 10 min before resuspension in PBS.

3.2.14.3 Adhesion Test:

- A mixture of (0.2ml) of the bacterial suspension, (0.2ml) of the epithelial cells suspension and (0.1ml) of PBS were incubated at (37°C) for one hr
- Unattached bacterial cells to uroepithelial cells were removed by centrifugation in PBS at 1000 rpm for (10min).
- Final pellet was resuspended in PBS then, a drop of was put on to a microscopic slide, air dried, fixed with methanol-acetic acid (3:1) and stained with methylene blue.
- The adhered bacterial cells to epithelial cells were observed by the Scanned with CamScanner

3.2.15 Effect of Concentrated Filtrates on Adhesion Property of *H.pylori*:-

Minimum inhibitory concentration of the concentrated filtrates of *L.acidophilus* isolate was used to investigate its effect on adherence factor property of *H.pylori* on uroepithelial cells in vitro as following:

Brain - heart infusion broth medium containing minimum inhibitory effect of concentrated filtrates was dispensed in sterile tubes and incubated with a loop full of liquid cultures of *H.pylori* (Hp1, Hp_2 and Hp3) isolates at (37°C) for (24hr) under microaerophilic conditions. Adhesion test as in (3.2.14.3) was repeated to examine inhibitory effect of concentrated filtrate after treatment.

3.3 Statistical Analysis:

Data were expressed as mean ± standard deviation and the statistical significances were calculated by ANOVA test and Chi square test.

The efficacy of the test was determined by calculating the sensitivity and specificity of each test.

Sensitivity was defined as the proportion of *H.pylori* infected which had a positive test and calculated as; [true positive / (True positive + False negative)] × 100.

Specificity was defined as the proportion of individual free of *H.pylori* which had a negative test and calculated as:

[True negative / (False positive + True negative)] × 100.

(Sim *et al;* 1995).

Chapter Four

Results and Discussion

4. Results and Discussion

4.1 Study Subject

4.1.1 Patient Groups

One hundred and thirty patients with dyspepsia included 41 females and 89 males, aging between (11-75) years and a mean of age 37.5 year. They underwent diagnostic upper gastrointestinal endoscopy at Endoscopy Department of Gastroenterology and Hepatology Teaching hospital and Baghdad Medical City in Baghdad, Iraq. Several gastric biopsy specimens were taken from the antrum and the body and they were subjected to various bacteriological and histological examinations. Fifty healthy individuals were also selected in this study. Sera were used for serological estimation.

4.1.1.1 Endoscopic Findings of Clinical States:

Results shown in (Figure 4-1) shows that, gastritis was endoscopically diagnosed in (28.5%) of the total patients, duodenal ulcer was seen in (16.1%) followed by (13.1%) for both peptic ulcer and the others who did not show any alteration at endoscopic examination (healthy), and gastric ulcer were diagnosed in (10%) of patients. While (4.6%) of patients had gastric erosion, (6.9%) suffering from duodenitis and (7.7%) of patients had gastric cancer.

Esophogastroduodenal (EGD) endoscopy permits gross visualization and localization of ulcerative lesions, mucosal nodularity associated with MALT lymphomas and other malignant lesions (Versalovic, 2003).

Similar results were obtained by both Al-Dhahar (2001) and Al-Hadi, (2001) who found high prevalence of gastritis among endoscopically diagnosed patients (41.5%), (38%) respectively.

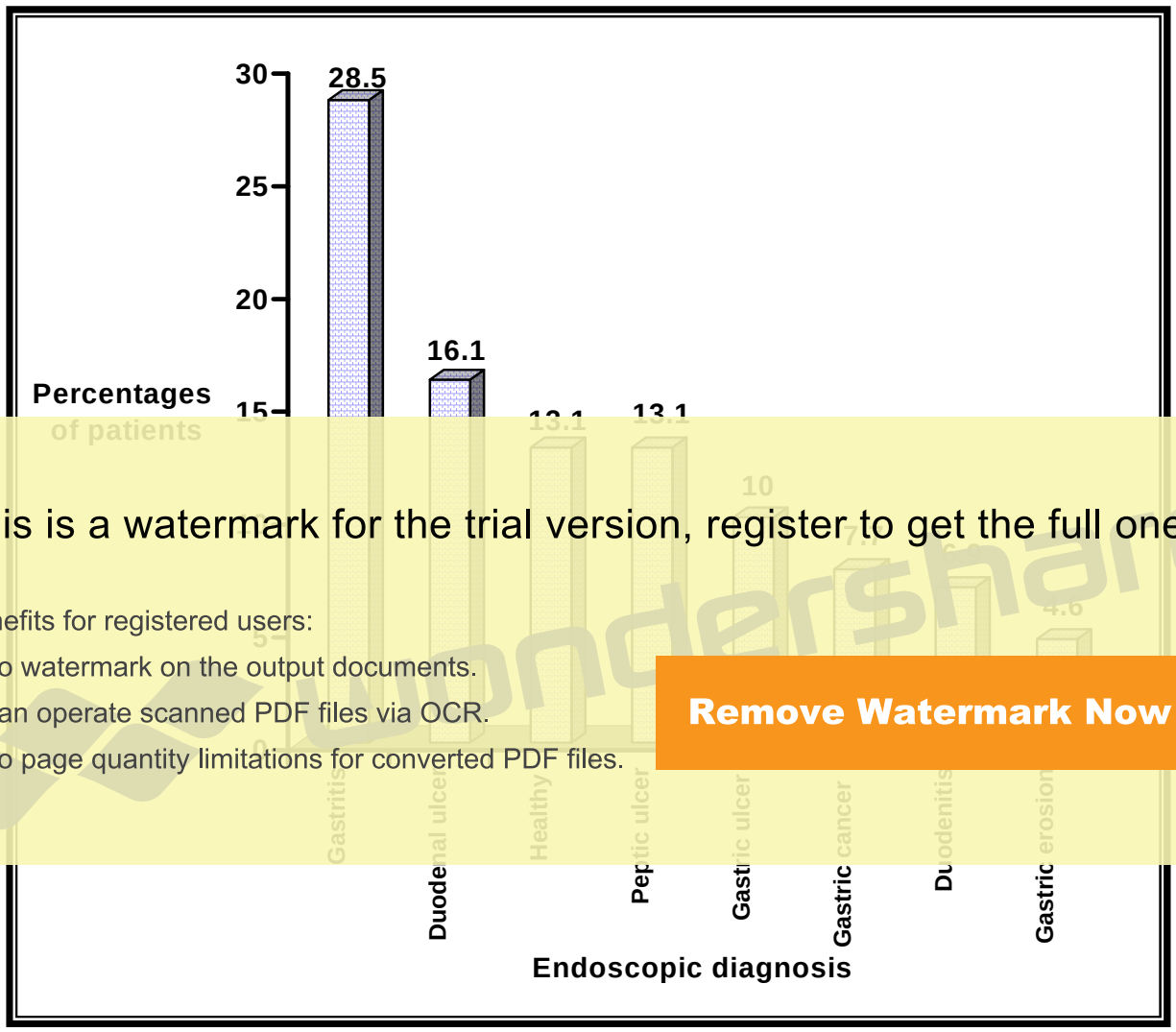


Figure (4-1): Percentages of Endoscopy Diagnosed Dyspeptic Patients.

Mohammed (2004) reported that gastritis was seen in (33.8%) among endoscopically diagnosed patients. While Hussein (2002) found that (30%) of duodenal ulcer patients were most prevalence among endoscopically diagnosed patients followed by gastritis in (20%) patients.

Weijmen *et al.*, (2001) found that oesophagitis was the most prevalent diagnosis (41.2%) during endoscopy while gastritis was observed in only a small minority (1.5%). Sepulveda *et al.*, (2005) indicated that performing an upper GI endoscopy is essential to establish a diagnosis of gastritis. Such endoscopic findings in chronic *H.pylori* infection may include areas of intestinal metaplasia so multiple biopsy samples are needed. Tissue sampling from both gastric antrum and corpus are essential to establish the topography of gastritis and to identify atrophy and intestinal metaplasia, which are patchy.

Sipponen *et al.*, (2000) recorded that endoscope with or without biopsy is reliable and efficient mean of diagnosing gastritis and atrophic gastritis but is invasive procedure. They also enable the determination of the topographic type and severity of gastritis and atrophic changes.

Some studies have suggested that *H.pylori* infection can be determined by unique endoscopic features particularly the presence of antral nodularity (Weijmen *et al.*, 2001).

4.1.1.2 Clinical Manifestation of *H.pylori* Infection:

Helicobacter pylori was detected in most of dyspeptic patients undergoing endoscopic examination. As shown in (Table 4-1), duodenitis shows highest percentage of infection (89%) when 8 out of 9 patients were found to be infected with *H.pylori*, followed by gastric erosion when 5 out of 6 patients (83.3%) show existence of *H.pylori*, duodenal ulcer when *H.pylori* was detected in 17 out of 21 patients (81%), and of gastritis when 28 out of 37 patients (75.6%) had positive results to it. Beside, (70%) of gastric cancer and peptic ulcer (70.6%) had *H.pylori* in 7 out of 10 patients and in 12 of 17 patients, respectively. Gastric ulcer was found to be infected with *H.pylori* in 9 of 13 patients (69%) while (23.5%) with normal endoscope appearance found to be infected with *H.pylori*.

Table (4-1): Correlation between Endoscope Findings and Presence of *H.pylori* Infection.

Endoscope Diagnosis	No. of cases	Infection	Case
		(No)	(%)
Duodenitis	9	8	89
Gastric erosion	6	5	83.3
Duodenal ulcer	21	17	81
Gastritis	37	28	75.6
Gastric cancer	10	7	70
Peptic ulcer	17	12	70.6
Gastric ulcer	13	9	69
Healthy	17	4	23.5
	130		

Similar results were obtained by other investigators such as Al-Dhahar (2001) and Hussien (2002) who recorded that (90.6%) and (86.7%) of duodenal ulcer patients were infected with *H.pylori* while it was found in (81.6%) and (80%) of gastritis patients, respectively. Also closed results were obtained by Hazell *et al*., (1987) which found that 88% of the gastritis cases having evidence of colonization by *H.pylori*. Velanvick (1996) on the other hand, found that (56.4%) of patients with duodenitis were infected with *H.pylori*, and Koike *et al*., (1999) mentioned that *H.pylori* infection was found in (33.7%) of patients with esophagitis. Versalovic, (2003) showed that areas of gastric metaplasia else where in gut, notably the duodenum, may become colonized with *H.pylori* thus setting the scene for ulceration.

Kanaghinis, (1995) mentioned that duodenal ulcer develops in areas of gastric metaplasia of the duodenal affected by *H.pylori* with chronic active duodenitis. He concluded that cytotoxic factors are related to *H.pylori* causing epithelial damage by special strains of *H.pylori* that are particularly ulcerogenic due to the gene encoding for the cytotoxic associated protein *cag* A and the antibodies to this protein increase in duodenal ulcer patients.

4.1.1.3 Detection the Occurrence of *H.pylori* in Dyspeptic Patients:

Several methods have been used to detect the presence of *H.pylori* such as; biopsy urease test, direct biopsy smear examination, histological and culture examination and serological evaluation by using ELISA test kit.

As shown in (Table 4-2), 104 of the 130 dyspeptic patients (80%) were positive by histological examination, 101 (77.7%), 85 (65%), 78 (60%) and 54 (41.5%) were positive by culture, biopsy urease test, Gram smear test and Giemsa smear test respectively. Furthermore, out of 75 patients (56%) were found to have high level of IgG specific antibodies to *H.pylori* infection.

Patients were considered to be infected with *H.pylori* if they were positive by culture and / or biopsy urease test, histology examination and demonstration of the organism microscopically either by Giemsa and / or direct Gram stains. Furthermore, 42 of 75 dyspeptic patients (56%) were considered to be infected with *H.pylori* when IgG specific antibodies titers were above the cut off value.

Accordingly 106 (81.5%) were considered to be infected in this study and 24 (18.4%) were negative using all tests. (Table 4-2), the use of multiple diagnostic methods is recommended to accurately diagnose *H.pylori* infection (Takio *et al.*, 1996).

Almost nearly results were obtained by Al-Any (2005) who found that prevalence of *H.pylori* was (83.3%) among 48 cases with peptic ulcer, and those by Hussien (2002) who found that (78%) of dyspeptic patients were infected with *Hpylori*.

Table (4-2): Diagnostic Tests of Dyspeptic Patient Biopsies for Detecting *H.pylori* Infection.

Tests	Positive Cases	Negative Cases	Total Cases	% of Positive Cases
Histology	104	26	130	80
Biopsy Urease Test	101	29	130	77.7
Biopsy Giemsa Stain Smear	85	45	130	65
Biopsy [illegible] Stain Smear	78	52	130	60
[illegible]	31	[illegible]	[illegible]	[illegible]
ELISA	42	33	75	56

In contrast, Al-Dhahar (2001) and Mohammed (2004) recorded that (74.8%) and (74.5%) of dyspeptic patients were found to be infected with *H.pylori* respectively.

Patients were considered to be *H.pylori* positive if two or more methods, whatever their nature, were positive, or if only culture or ELISA test was positive, as no false-positive results are expected for culture (Querroz *et al.*, 1999). Megraud *et al.*, (1999) found that gastric biopsy specimens are necessary for direct tissue diagnosis of *H.pylori* by rapid urease test or histology.

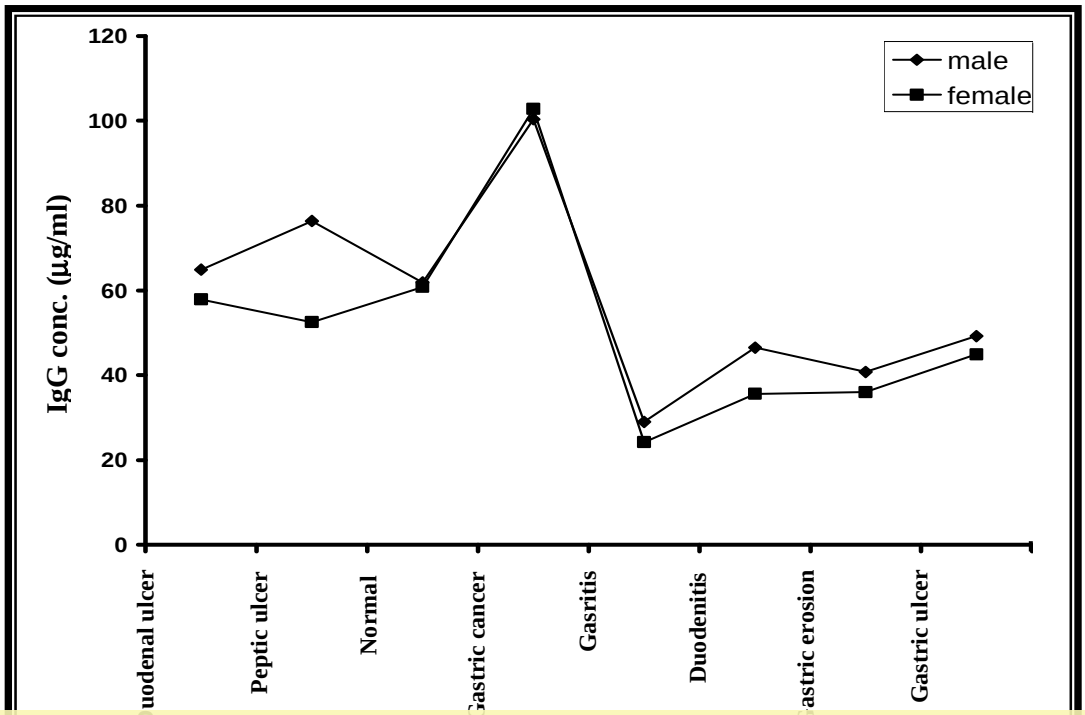
Two positive biopsy tests were used as the gold standard to determine the sensitivity and specificity for each test; these were histological examination of gastric biopsy specimens and biopsy urease test. Endoscopy finding indicated that urease test is the first choice test on an antral biopsy. However, if a biopsy urease negative, *H.pylori* infection may be diagnosed by histological or serological procedure (Blaser *et al.*, 1997).

4.1.1.4 ELISA Examination:

Serological determination of antibodies specific to *H.pylori* is simple and can be performed in any clinical laboratory. Rathbone *et al.*, (1986) found that ELISA techniques may be used to show a significant rise of IgG specific antibodies to *H.pylori* in patients with histological evidence of the organism.

In this study, *H.pylori* specific IgG concentration was determined by ELISA method and among (75 dyspeptic patients and 15 healthy controls). The precrete cutoff value (provided manufacturer) were applied. The variable detected is an abnormally distributed variable with mark positive values as shown in (Figure 4-2). Therefore, the mean and standard error will be presented as descriptive statistics for this variable in different groups to observe differences between groups.

The level of total serum immunoglobulin show significant differences in mean of IgG concentration between gastric cancer and control cases. In addition, significant elevation was observed in the mean values of duodenal ulcer cases and control with (P-value ≤0.01). While no significant differences was obtained in the mean of IgG concentration of gastric ulcer, gastritis, duodenitis, peptic ulcer, gastric erosion and that of control, although higher level of the mean IgG concentration was obtained among them comparison with controls but it failed to reach the level of significant.



Diagnosed of Dyspeptic Patients.

Figure (4-2): Frequency Distribution of Dyspeptic Patients by *Helicobacter* Specific IgG Measured by ELISA Method

be detected in the serum level of specific IgG *H.pylori* antibodies. While no significant differences were revealed among males and females of other groups.

Present results were agree with those obtained by Abdalla, (2002) who indicated that when (77.5%) of the females were seropositive which is almost equal to the (77.8%) of seropositively seen in the male gander. But disagreed with Al-Dahar, (2001) who found that the means of IgG titers showed high level compared to the normal values, and statistical analysis show that there are significant differences between patients and control in IgG titers. Furthermore Al-Rawy, (2005) found that seroprevalence of *H.pylori* infection was significantly higher in men than in women. Banatvala *et al.*, (1994) found that there was an insignificant tendency towards higher prevalence of *H.pylori* infection in man than in women.

Al-Ani, (2005) found that the presence of *H.pylori* corelated with statistical significant higher mean IgG concentration compared to those with no such evidence. Rathbone *et al*., (1985) reported a considerable raised IgG and IgA serum antibody titers in patients from whom *H.pylori* was cultured. Wulffan *et al*., (1988) also pointed that patients who had *H.pylori* associated gastritis suffered from significant elevation of *H.pylori* specific IgG. These results may explained by the fact that *H.pylori* is chronic infection.

4.1.1.5 Laboratory Investigation of Biopsy Specimens:

4.1.1.5.1 Biopsy Urease Test (BUT):-

The biopsy urease test is a simple and cheap alternative one that can be performed at the bedside. *H.pylori* produces such abundant urease that its action can be detected directly in biopsy specimens (Skirrow, 1990). According to (Table 4-3), (77.7%) (14 out of 30) of the dyspeptic patients were positive using biopsy [illegible] specimens gave positive results within result after one hr. and 40 cases after (24hr). A false result obtained when 7 noninfected persons gave positive result after (24hr). This may due to contamination by other organisms with urease activity.

Color change from yellow to pink with CLO test indicates the presence of *H.pylori*. It has been shown that *H.pylori* urease remains active in an acid medium whereas other commonly found bacterial (e.g. from *Proteus*, *Pseudomonas* and *E.coli*) were inactive.

Hazell *et al.*, (1987) reported that the time for a positive result is usually proportional to the number of *Helicobacters.* This means, when a positive result is obtained rapidly it is likely that the *Helicobacter* count is high and more organisms seen in the biopsy, which is more likely a positive biopsy urease test within (30min). Therefore, urease test is used to test the relative numbers of *H.pylori* present at any particular site in the stomach.

Table (4-3): Biopsy Urease Test Results at Different Period.

Gastric Biopsy	Time	Result	
		Positive	Negative
Infected	Within 30 min.	23	107
	After 1 hr.	38	92
	After 24 hr.	40	90
Total		101	
Non infect	Within 30 min.	-	29
	After 1 hr.	-	29
	After 24 hr.	7	22

Five of infected patients gave negative biopsy urease test but positive by culture and histological examination, so they were considered as false negative results. These negative results may be due to the presence of a low bacterial density or to recent use of antibiotics, bismuth, or PPI which may render rapid urease test false negative (Bermejo *et al.*, 2002). It may also due to patchy distribution of *H.pylori* colonization of the affected areas of gastric mucosa which is heavily colonized areas adjacent to those with no colorization (Hazell *et al.*, 1987). Also false negative urease test could occur in patients infected with urease negative *Helicobacte*r in the cases of gastritis (Pena *et al.*, 2002).

When sensitivity and specificity of biopsy urease test for detecting *H.pylori* infection were estimated as shown in (Table 4-4), a great significant association was found between *H.pylori* infection and biopsy urease test (x^2 = 59.9, P = 0.000001, CI = 95%) with sensitivity and specificity (95%), (72%), respectively, and positive predictive value of (92%).

Table (4-4): Sensitivity and Specificity of Biopsy Urease Test for Detecting *H.pylori*.

Test of significant	Value
Chi-Squire test.	59.94
P-value Screening [95% CI].	0.00000
Prevalence.	78 %
Sensitivity.	95 %
Specificity.	72 %
Accuracy.	90 %
Predictive value of (+ve) result.	92 %
Predictive value of (-ve) result.	81 %

Sensitivity of detection depends on organism load in mucosal biopsy specimen and number of biopsy samples. Optimally, biopsy samples of the corpus and antrum should be obtained for rapid urease testing.

Results of present study agreed with those obtained by Al-Dhahar (2001) who found that biopsy urease test has a sensitivity of (91%) and a specificity of (93.3%). Mohammed (2004) also found that the sensitivity and specificity of urease test were (87.2%), (86.6%) respectively.

Nearly results were indicated by Cutler *et al.*, (1995) and Onders (1997) which indicated that the sensitivity of biopsy urease test were (89.6%), (92%) respectively. Cutler *et al.*, (1995) indicated that rapid urease test have specificity and sensitivity of greater than (90%).

It was found that using two biopsies (one from gastric antrum and one from gastric body) enhances the value of biopsy urease test in patient assessment (Hazell *et al*., 1987).

Exceptional urease activity of this bacterium is in order of 100 times greater than *Proteus vulgaris*, which means that its detection in gastric tissue can be made directly by passing the need for culture (which requires freshly prepared media with a humid microaerophilic environment up to 5 days) to obtain the result (Marshall *et al*., 1987).

4.1.1.3.2 Direct Biopsy Smear:

Helicobacter pylori lies within the mucus layer of the gastroduodenal mucus (Sarosiek *et al*., 1991). During upper gastrointestinal endoscopy, four gastric biopsy specimens (two from each of antrum and body) were applied to diagnose the presence of *H.pylori* infection by direct biopsy smear method. Versalovic, (2003) noted that diagnostic confirmation the presence of *H.pylori* necessitates biopsy sampling of the gastric corpus and antrum due to patchy distribution of *Helicobacter* within mucus layer, and as a result, multiple biopsy may be necessary for diagnosis.

In this test out of 130 dyspeptic patients, 85 (65.3%) gave positive result for the presence of *H.pylori* using Giemsa stain and 78 (60%) with Gram stain as shown in (Figures 4-3 and 4-4).

Stained smears of biopsy material provide an opportunity for rapid evaluation of *H.pylori* status, Gram stain, modified Gram stain with carbol fuchsin as counter stain and Giemsa stain, make *H.pylori* visible and allowing morphology of the bacterium to be determined. The sensitivity of these tests, however, is largely dependent on the number of bacteria present in the biopsy material (Price *et al*., 1997).

(Table 4-5) shows the distribution of *H.pylori* by using Giemsa stain method which was recognized in (63) out of (76) gastric antrum (83%) of the infected patients and in 37 (63%) when Gram stain was used. In contrast, only (22) out of (54) infected gastric body patients (40.7%) show the presence of *H.pylori* by Giemsa stain and in (41) out of (71) infected patients (58%) by Gram stain. Giemsa stain method can be used to enhance the visibility of bacteria in the specimen and it appears [illegible] (Montgomory *et al*., 1988).

David *et al*., (2000) found that the density of *H.pylori* in the antrum of duodenal ulcer is greater than in the antrum of patients with *H.pylori* gastritis and, density of *H.pylori* is higher in the corpus of patients with *H.pylori* gastritis than in those with duodenal ulcer, suggesting that acid secretion plays a critical role in these phenomena. With Gram and Giemsa stains, a relatively poor contrast exists between organisms and background stained material. Heat fixed touch preparation of biopsy material stained with Gram stain using safranine counter stain showed (72%) sensitivity and (100%) specificity (Montgomery *et al*., 1988).

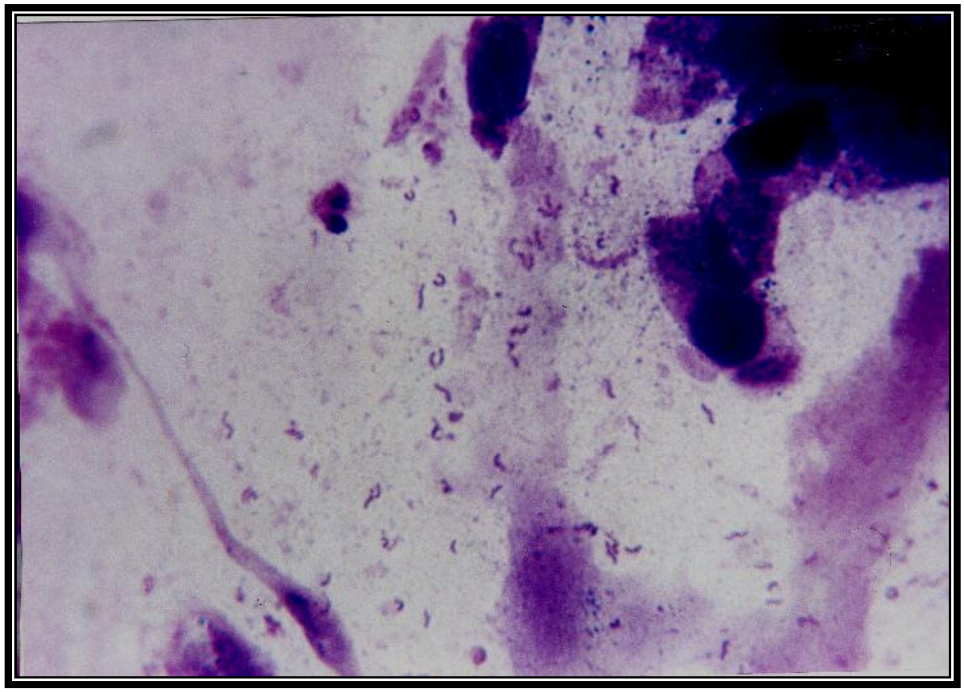


Figure (4-3): *Helicobacter pylori* of Direct Biopsy Smear Stained

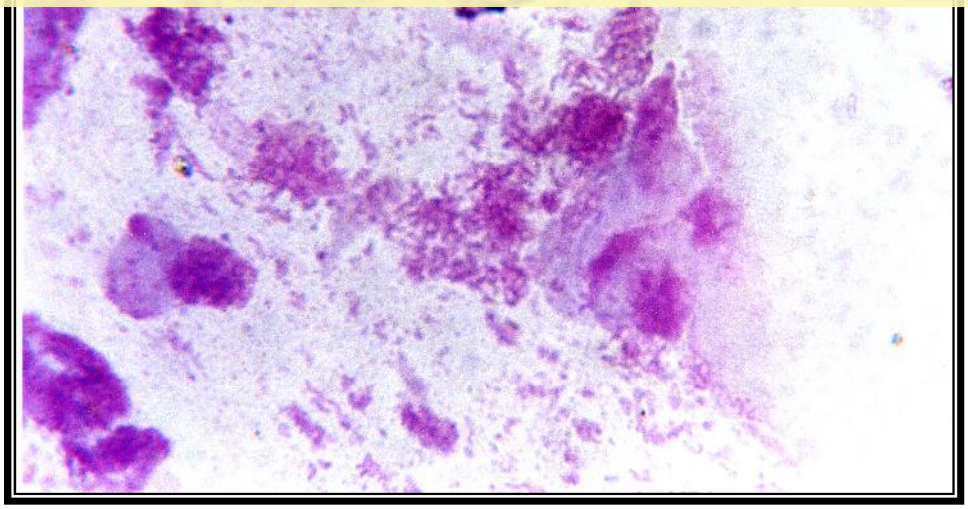


Figure (4-4): *Helicobacter pylori* of Direct Biopsy Smear Stained by Gram - Stain (100xs).

Table (4-5): Direct Biopsy Examination of Gastric Antrum and Body for *H.pylori* Distribution.

Site of Infection	Giemsa Stain				Gram Stain			
	Positive		Negative		Positive		Negative	
	No.	%	No.	%	No.	%	No.	%
Antrum	63	83	13	17	37	63	22	37
Body	22	40.7	32	59.3	41	58	30	42
Total	85		45		78		52	

specimens with a positive predictive percentage of (78%) as compared to the Gram stain with sensitivity of (90%) and (67%) positive predictive value.

Results of the present study are similar to those of Parsonnet *et al.*, (1988) who found that sensitivity of Gram stain was 100%. Moreover, McNulty *et al.*, (1988) reported that this test was even more sensitive than the urease test. Nearly results were also obtained by Klelkar *et al.*, (1990) which found that detection the *H.pylori* infection by Gram stain is very useful, as it is quick, cheap, sensitive and specific with sensitivity of (96.4%) and specificity (96.3%). They also indicated that Gram stain is a simple relative inexpensive and faster method than the histological method. It is highly sensitive and specific.

Table (4-6): The Sensitivity and Specificity of Giemsa and Gram Stain Smear Test.

Test of significant	Giemsa test value	Gram test value
Chi-squire	33.19	10.10
P-value	0.000001	0.0014
Screening 95% CI		
Prevalence	65	60
Sensitivity	95	90
Specificity	49	35
Accuracy	79	68
Predictive (+ve) value	78	67
Predictive (-ve)value	85	69

use of an appropriate stain (Peterson and Graham, 2001). In contrast, Morris *et al.*, (1989) and Al-Baldawi (2001) found that sensitivity of direct biopsy smear were (78.6%) and (69.2%), respectively.

Furthermore, present results disagreed with those obtained by Simor *et al.*, (1990) who found that the sensitivity and specificity to visualize *H.pylori* were (67%) and (98%), respectively.

4.1.1.5.3 Histological Examination of Gastric Biopsy:-

The advantage of histologic diagnosis is through confirmation of active infection as well as an evaluation of mucosa for the presence of potential associated pathogenic state acute/chronic gastritis, atrophy, metaplasia, dyspepsia, gastric lymphoma and malignancy (Sepulveda *et al.*, 2005).

(Table 4-7) shows that active gastritis and active superficial gastritis were found in 32% (42) and in 9% (12) of the dyspeptic patients, followed by chronic gastritis, chronic superficial gastritis and chronic atrophy gastritis with 16% (21), 13% (17) and 10% (13), respectively.

Table (4-7): Histological Diagnosis of Dyspeptic Patients in Association with *H.pylori* Infection.

Histological Aspect	Occurrence		Infection with *H.pylori*	
	No.	%	No.	%
Active gastritis	42	32	41	98
Active superficial gastritis	12	9	11	92
Chronic gastritis	21	16	20	95
Chronic atrophy gastritis	17	13	14	82
Chronic superficial gastritis	13	10	11	85
Gastric lymphoma	6	5	3	50
Gastric adenocarcinoma	5	4	3	60
Healthy	14	11	10	71
Total	130	100	104	

Adenocarcinoma and lymphoma were seen in 4% (5) and 5% (6) of the cases. On the otherhand, normal gastric biopsies were seen in 11% (14) of dyspeptic patients. Chronic gastritis was found to be the commonest histological finding in the gastric biopsies of dyspeptic patients (Figure 4-5).

Results detected in this study agree with those obtained by Mohammed (2004) who found that chronic gastritis was detected in (40.6%) of cases. While Hussien (2002) reported that chronic gastritis were seen in (80%). Close results were obtained by Hussien (2002) and Al-Janabi (1992) who indicated that adenocarcinoma was seen in (4%) and (2%) of the cases, respectively. In contrast, Testonip *et al.*, (1995) found that adenocarcinoma was seen in (42.9%) cases and in (79%) by El-Zimaity *et al.*, (1999).

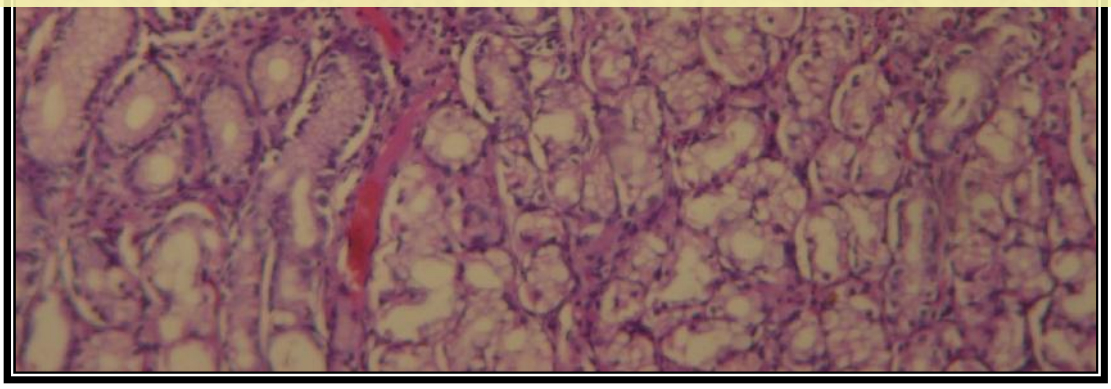


Figure (4-5): Histological Sectioning Shows Chronic Superficial Gastritis (40xs).

Helicobacter pylori was detected in 34 of 42 cases with active gastritis (81%), in 11 out of 12 cases with active superficial gastritis (92%), 20 out of 21 with chronic gastritis (95%) and in 12 out of 17, 11 out of 13 cases with chronic atrophy (70.5%) and chronic superficial gastritis (85%), respectively (Table 4-7).

Theses results closely resemble those obtained by Mohammed (2004) who found that *H.pylori* was present in (86.4%) of cases with chronic gastritis as well as Langenberg *et al.*, (1984) and Bahiya *et al.*, (1995) indicated that (96.8%) and (95.4%) of chronic gastritis cases were caused by infection with *H.pylori.*

In this study, gastric lymphoma and adenocarcinoma were found to be infected with *H.pylori* in (50%) and (60%) of cases. Similar results were obtained by Mohammed (2004) which found that *H.pylori* were present in (58%) and (50%) of adenocarcinoma and gastric lymphoma cases respectively. While G[illegible] due to the difficulty to recognize these microorganisms in malignant glands due to the changes in composition and secretion of the glands (Asaka *et al.*, 1997).

Sipponen and Stolte, (1997) suggested that *H.pylori* are present in gastric biopsies of other areas away from the malignant ones. Normal gastric biopsies could be associated with *H.pylori* infection. In this study 10 (71%) normal gastric biopsies out of 14 cases revealed the presence of *H.pylori*.

Goodwin *et al.*, (1985) found that (6.3%) of the normal biopsies were infected with *H.pylori* organisms, and Buck *et al.*, (1986) found its presence in (14%) of normal biopsies.

These observations could also be attributed to recent infection, or patchy involvement, pathogenicity might be present in gastric region other that invaded by the bacteria (Marshall and Warren, 1984). This test was considered to be gold standard for its highly specificity and sensitivity (100%).

Both of Giemsa and Haematoxylin and Eosin were used to identify *H.pylori* organisms and assert the severity of histological gastritis in paraffin wax section of fixed antrum and corpus biopsies, as shown in (Figures 4-6, 4-7).

Curved shape or undulating rods stained blue in Giemsa stain were present in histological sections of gastric biopsy specimens while considered to be as *H.pylori* following (Marshall and Warren, 1984; Govosol *et al*., 1995).

Al-Jalili, (1996) mentioned that histological examination of Giemsa stain of gastric biopsy specimens [illegible] with those of Al-Jalili, (1996) who found the sensitivity and specificity of Giemsa stain were (100%). Luthra *et al.*, (1998) found that histology has a sensitivity of (99.1%) and specificity (99.5%) respectively.

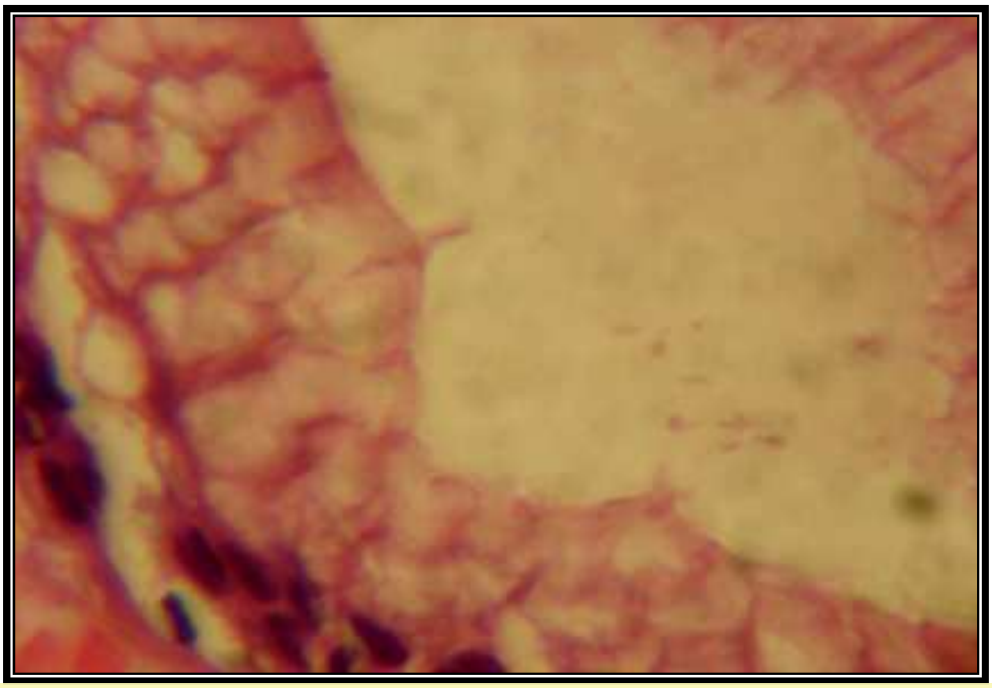


Figure (4-6): *Helicobacter pylori* of Gastritis Antral Biopsy Specimens Stained by Giemsa Stain (100xs).

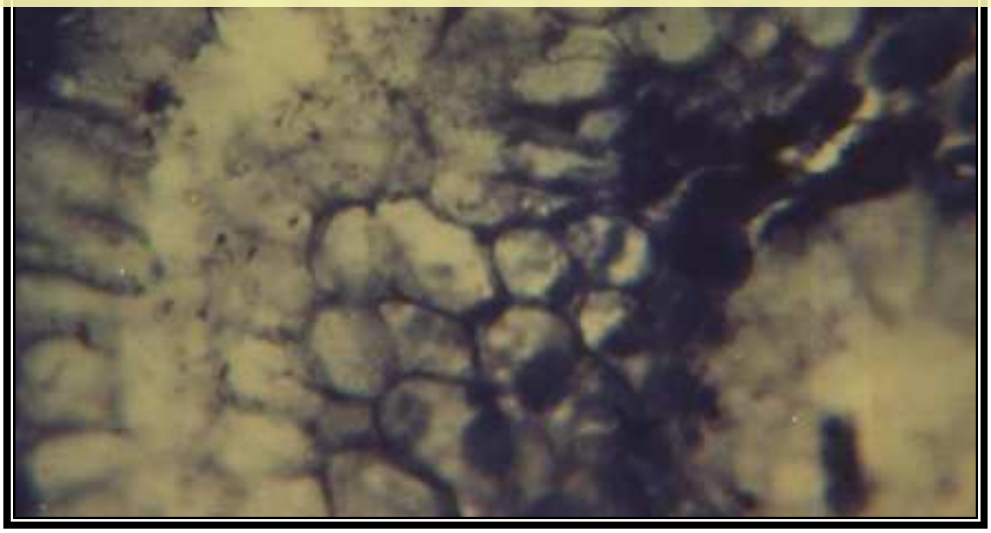


Figure (4-7): ***Helicobacter pylori*** **of Gastritis Antral Biopsy Specimens Stained by Giemsa Stain (100xs).**

4.1.1.5.4 Culturing of *Helicobacter pylori*:-

Although various methods have been developed for detecting *H.pylori* infection, bacterial culture remains extremely important. Isolation of *H.pylori* enables susceptibility testing, which predicts the like hood of eradication. *H.pylori* is fastidious organism so various factors, including bacterial density, transport conditions, culture medium and microaerophilic condition, directly influence the yield of culture (Siu *et al.*, 1998).

Table (4-8) shows presence of *H.pylori* in the stomach of dyspeptic patients, as determined by endoscope in a correlation with the culture results. *H.pylori* was isolated from 6 of 9 gastric ulcer patients (66.6%), 7 (41%) of duodenal ulcer patients, 5 of 12 peptic ulcer patients (41.6%), 10 of 28 gastritis patients (35.7%), 2 (28.5%) of gastric cancer patients and one of 4 healthy individual (25%). While *H.pylori* was not isolated from any duodenal ulcer (5 patients). Graham *et al.*, (1987) mentioned that about half of the patients undergoing upper gastrointestinal endoscope and *H.pylori* infection of the stomach has been associated with gastric ulcer, duodenal ulcer and gastritis.

Similar results to the present study were obtained by Giupczynski *et al.*, (1988) who found that the prevalence of *H.pylori* was (64%) in gastric ulcer and (80%) in duodenal ulcer. Marshall and warren (1984) stated that *H.pylori* is the major etiological agent of gastritis due to detection of *H.pylori* among gastritis patients in the absence of other organic disease.

Almost similar results were obtained by McColl, (2000) which found that (20-50)% of dyspeptic patients with a positive *H.pylori* test have evidence of underlying gastric ulcer or duodenal ulcer.

Helicobacter pylori, which is only colonized gastric type epithelium, may cause local damage within the duodenum. This was explained by the presence of patches of gastric metaplasia in the duodenum of duodenal ulcer patients. It was found that *H.pylori* may cause ulcer by provoking inflammation or by releasing an ulcerogenic toxin.

In contrast, Stephen *et al.*, (1996) indicated that *H.pylori* is present in over (90%) of patients with gastric ulcer and it is the main causes of duodenal and gastric ulcer.

Marshall and Warren, (1984) noted that using endoscope evaluation (55%) of patient with gastritis were colonized by *H.pylori*. While McNulty and Watson, (1984) found that *H.pylori* associated with (77%) of gastritis cases.

Table (4-8).Culturing Results of *H.pylori* Isolated from Endoscopically Examined Dyspeptic Patients

Diagnosis	No.	Isolated (No.)	*H.pylori* (%)
Gastritis	28	10	35.7
Duodenal ulcer	17	7	41
Peptic ulcer	12	5	41.6
Gastric ulcer	9	6	66.6
Duodenitis	8	-	-
Gastric cancer	7	2	28.5
Gastric erosion	5	-	-
Healthy	4	1	25
Total	90	31	

As well as Jones *et al.*, (1984), reported that up to (90%) of patients with gastritis were colonized with *H.pylori*.

Culturing on solid media is the standard technique used in most of the laboratories for isolation of *H.pylori* from gastric biopsy specimens (Marshall *et al.*, 1984).

A variety of media, selective and nonselective, or a combination of both has been proposed for use in the primary isolation of *H. pylori.* A total of 520 gastric mucosal biopsy specimens were obtained (two pieces) from each of antrum and body of 130 dyspeptic patients undergoing endoscope, by using selective and nonselective media. *H.pylori* was detected in 31 patients only, yielding an isolation rate of (24%). None of the media by itself gave maximum recovery rate. Brain - heart infusion agar was the most sensitive culture medium for isolation of *H.pylori* among dyspeptic patients with (recovery rate = 74%) followed by Columbia agar (18.5%), Brucella agar (15.4%) and non selective media Chocolate agar (14.6%),

As shown in (Figure 4-8), these last finding illustrated in (Table 4-9), which showed that among (130) total cases, (99) were given negative Brain - heart infusion agar growth and only 15 of them were positive by Columbia agar, 8 by Chocolate agar and (5) by Brucella agar while only (3) were positive by Blood agar. On the other hand, among (31) cases with *H.pylori* positive culture only (15, 12, 9 and 5) cases were positive with Brucella agar, Chocolate agar Columbia agar, and Blood agar, respectively.

Culturing was the least sensitive when only 31 (30%) of the positive cases were detected despite meticulous care in the whole steps of culturing including careful media preparation, transport, incubation, atmosphere and identification steps.

Al-Hamadani (2000) and Hussien (2002) found that the isolation rates of *H.pylori* were (19.4%) and (14%), respectively. While Al-Dhahar (2001) and AL-Hadi (2001) found that *H.pylori* isolation rate, were (50%) and (49.4%), respectively. In contrast, Al-Baldawi (2001), Mohammed (2004) and Al-Ani (2005) found that the recovery rate of *H.pylori* were (70.8%), (74.5%) and (79.2%), respectively.

This may be due to the fastidious nature of *H.pylori* and to a number of factors that are hard to control (patchy distribution of the organism on the gastric mucosa, contamination of biopsy forceps, ingestion of anesthetia, presence of oropharyngeal flora, loss of viability of the organisms during transportation) and that are, altogether responsible for a poor negative predictive value associated with culture of *H.pylori*.

Piccolomini *et al.*, (1997) stated that practical contamination is difficult to avoid and, therefore, a suitable selective media with antibiotics are usually essential to detect *H.pylori*. Results of the present study is agree with that found different [illegible] *H.pylori* as compared with other culture media followed by Columbia agar. On the other hand, Chocolate and Blood agar showed lowest rate of recovery for *H.pylori*.

Piccolomini *et al.*, (1996) recorded that nonselective media yielded the lowest rate because of the abundant growth of contaminants (especially *Proteus* spp., *Pseudomonas aerugenosa*, *Strptococcus* spp. and *Candida* spp.) that obscured the growth of *H.pylori*. So growth of *H.pylori* could not be detected on the plate in presence of a high number of contaminants. Similar results were stated by Hachem *et al.*, (1995) who found that the recovery rate of *H.pylori* using Brain-heart infusion agar was (96%) while Piccolomini *et al.*, (1997) found recovery rate by using Brain heat infusion agar as (35%).

Table (4-9): Results of Selective and Nonselective Media which Used for Isolation of *H.pylori*.

Culture media and results	Results of Culture *BHI- Agar Media		Total
	Negative	Positive	
* Columbia Agar			
Negative	84	22	106
positive	15	9	24
Total	99	31	130
* Brucella Agar			
Negative	94	16	110
Positive	5	15	20
Total	99	31	130
Chocolate Agar			
Negative	92	19	111
Positive	7	12	19
Total	99	31	130
Blood Agar			
Negative	96	26	122
Positive	3	5	8
Total	99	31	130

*** Selective Media: Contain (10µg/ml) vancomycim, (10µg/ml) amphotricin, (5µg/ml) polymyxin and (5µg/ml) trimethoprim with (7%) horse blood.**

Ansory *et al.*, (1991) noted that failure of *H.pylori* to grow from appropriate specimens may be due to cimetidine tablets ingested before endoscope could be provide a sufficiently high concentration of drug to, at least partially, inhibit *H.pylori*. Antibiotics, bismuth preparation, inadequate specimens or failure in the microbiological technique all can lead to failure in culturing *H.pylori*. Besides, gastric mucosa can be colonized by organisms other than *H.pylori* if the gastric pH is raised due to H2-anatogonist, or hypochlorihydria. These organisms may then obscure the growth of *H.pylori*. The endoscope will be contaminated by oral bacteria during passage through orpharynx, and by Gram - negative when duodenum is examined (Roosendeal *et al.*, 1995; Vander *et al.*, 1996).

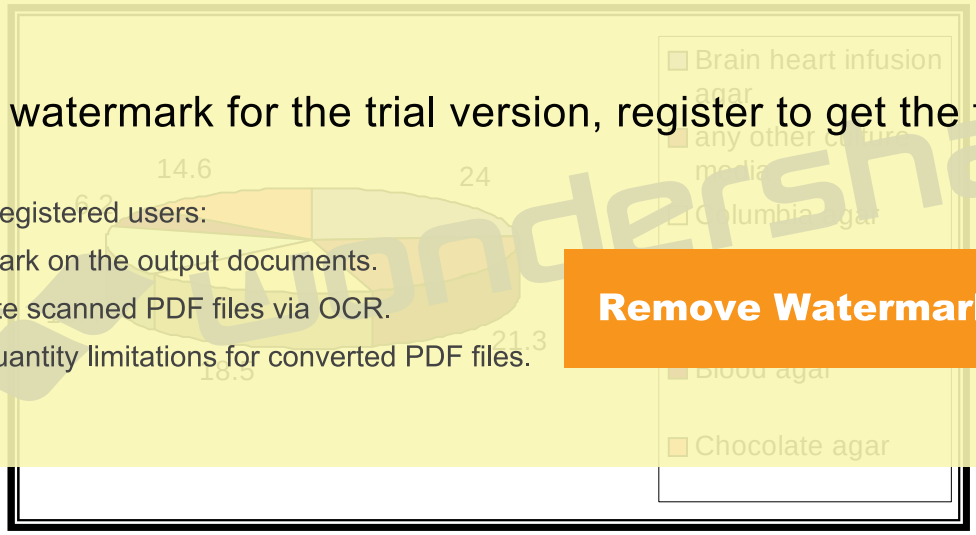


Figure (4-8): Recovery Rates of *H.ylori* on Different Culture Media Among Dyspeptic Patients.

Contamination mainly occurs in the theatre. Improper sterilization of biopsy forceps is one important cause. This is usually done using (2%) cidex for (10) minutes the risk of contamination from food in the stomach could be a minor case because most patients, are well prepared before endoscopy (Cotton and Williams, 1996). The second source of contamination could be due to blood used in culture media. This is obtained to be from horse. The procedure is task difficult and not free from the risk of contamination.

(Table 4-10) shows that the sensitivity of culture is (26%), while its specificity is (100%), and this is in agreement with Goodwin (1985) who found the sensitivity of culture was (50%). Also Orders (1997) found that the sensitivity of culture was (42%) and indicated that culture results showed a high rate of false negatives, which is consistent with the organisms over all fastidious nature that makes it extremely difficult to culture. Al-Janabi (1992) found the sensitivity of culture (42%), while Al-Jalili (1996) found the sensitivity of culture (73.6%) and specificity of (100%).

Table (4-10): The Sensitivity and Specificity of Culture Media.

Test of significant	Value
Chi-square	11
P-value	0.0014
Confidence [95%]	[ ]
Prevalence	
Sensitivity	26 %
Specificity	100 %
Accuracy	44 %
Positive predictive value	30 %
Negative predictive value	100 %

4.1.1.6 Identification of *H.pylori* Isolates:

Culture examination of *H.pylori* appeared colonies after (3-5) days of plating on Brain - heart infusion agar and between (7-10) days on other selective media used under microaerophilic conditions at (37°C). The colonies were tiny, glistering, translucent and convex with entire edges as shown in (Figure 4-9).

Figure (4-9): Colonies of *H. pylori* on Brain - Heart

technique, cells appeared as slightly curved or straight rods to curved bacilli with Gram - negative reaction. Extended cultivation to (10-15) days changed morphology of the bacteria from a helical form to a coccoid one. (Figs. 4-10 and 4-11). Catrenick and Makin (1991) mentioned that changing bacterium morphology from helical to coccoid may occur under various conditions such as extended cultivation, aerobic culturing, alkaline pH and antibiotic treatment.

Furthermore, morphology of *H.pylori* which is observed in gastric biopsies may differ markedly from that observed in a Gram stained preparation of organisms. It usually appeared as a slightly curved or straight rods whereas stained tissue biopsy specimens usually reveal a helical or more curve appearance (Fox, 1997).

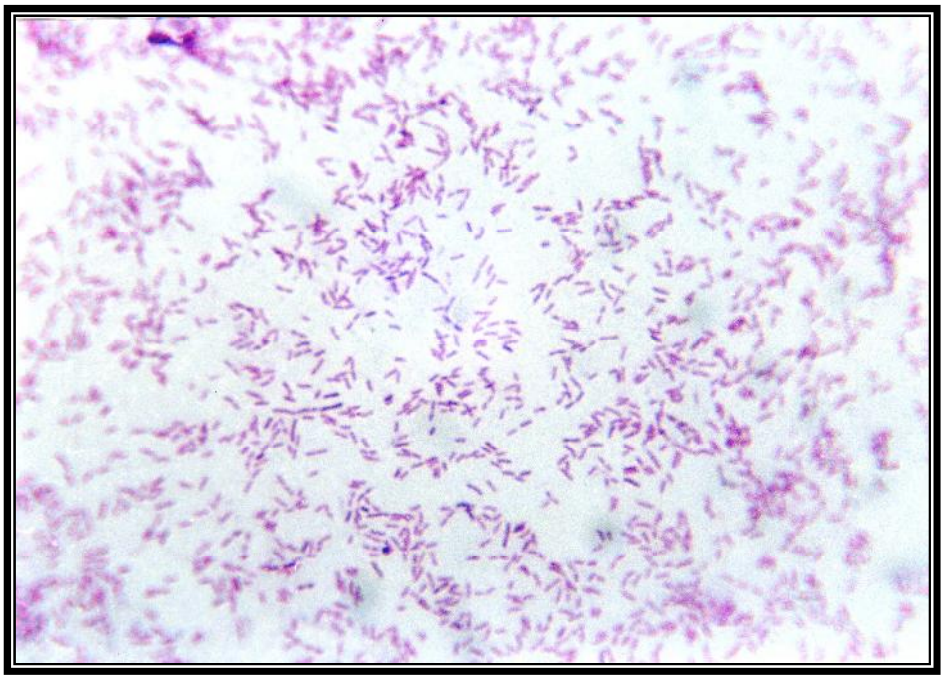


Figure (4-10): Gram Stain of *H. pylori* after 5 Days Culturing on Brain - Heart Infusion Agar (100xs).

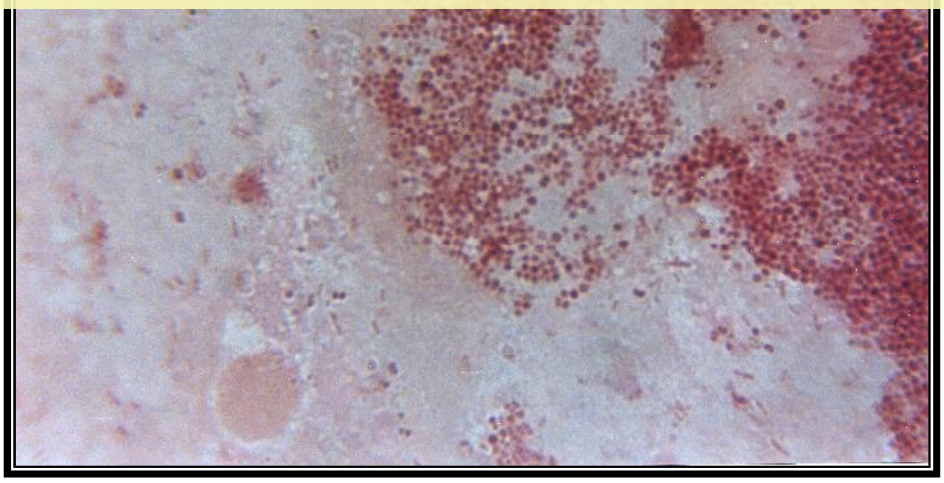


Figure (4-11): Gram Stain of *H. pylori* after 15 Days Culturing on Brain - Heart Infusion Agar (100xs).

Baron *et al.*, (1994) stated that different diagnostic methods are used for detection of *H.pylori*. As shown in (Scheme 4-1) once *H.pylori* is cultured, it may be identified by colony morphology, staining and positive (urease, catalase and oxidase) tests.

H.pylori produces urease at a very high activity through color changing from yellow to pink after few minutes, few hours and after (24hr) (Quirioz *et al.*, 1999).

Dunn *et al.*, (1990) reported that urease is found in the cytoplasm and on the membrane of *H.pylori* cells. Compared to other urease positive microorganisms, *H.pylori* produces larger quantities of highly active urease.

Isolates of *H.pylori* were also catalase positive through air bubbles production and oxidase positive through color changing from violet to dark violet on the filter paper of suspected isolates. Moreover, good growth was obtained on Campylobacter agar while no g[illegible] at (25 C) and (45 C) incubation temperatures.

Results of susceptibility test to nalidixic acid and cephalothin indicated that approximately all isolates of *H.pylori* showed resistance to nalidixic acid but susceptible to cephalothin.

4.1.1.7 Antibiotic Sensitivity of *Helicobacter pylori*:

Infections with *H.pylori* strains resistant to clarithromycin or metronidazole have been associated with a greater incidence of treatment failures than infections with susceptible strains thus various susceptibility testing methods such as broth microdilution, disk diffusion, the E test and agar dilution have been used to assess antimicrobial resistance in *H.pylori* (Calvet *et al.*, 2000; Koboyaski *et al.*, 2004).

Standard disk diffusion method was used to determine the antibiotic resistance pattern of rapid urease producing *H.pylori* isolates against (12) different antibiotics, as shown in (Table 4-11). Generally, a vast of resistance was detected among *H.pylori* isolates against the antibiotic used. Among them, no single antibiotic was resisted by all the isolates of *H.pylori* or sensitive to them.

It was found that the more effective antibiotic against *H.pylori* isolates was ciprofloxacin (from quinolons groups) when (8) isolates were sensitive to it, while only (5) isolates were resistant. Followed by erythromycin and clarithromycin (of macrolide groups) when (7) isolates were resistant and the other (6) isolates were sensitive to them. Adversely, the less effective antibiotics were pencillin G and ampicillin (from beta lactam group) when all (except [illegible] isolates were resistant to them. Followed by [illegible] with only (3) isolates sensitive while the other remaining [illegible] were resistant. Followed by metronidazole (nitrometadazole group) and cefrioxme (cephalosporin group) with only four isolates were sensitive to them while the remaining (9) isolates were resistant. Then amoxicilin when (8) isolates were resistant and the other (5) isolates sensitive. However other antibiotics were distributed between these ranges as shown in (Table 4-11).

Development of antibiotic resistance may be explained by different mechanisms; It may either involve a modification in DNA gyrase, the target enzyme of quinolones, or due to a modification in the bacterial outer membrane proteins, rendering the drug unable to penetrate inside the bacteria (Glupczynski and Buette, 1990). Also it was found that in patients heavily infected with *H.pylori* ($\geq 10^9$ colonies at some sites of infection),

spontaneous mutation may be selected for few resistant mutants which would subsequently replace the susceptible population of bacteria. This selection of resistant organism may be further facilitated by low concentration of drug in some areas of the colonized stomach and by reduced antibacterial activity in the presence of a low pH (Taylor and Courvalin, 1988).

McNulty *et al.,* (2002) discovered that differences in susceptibility were found in isolates obtained from both corpus and antrum patients, the discrepancies may be due to co - infection in an individual with two different strains of distinct linage.

Frequency of resistance *H.pylori* isolates was also estimated in this study as shown in (Figure 4-12). Ciprofloxacin has the lowest percentage (38%) when only (5) isolates were resistant to it, followed by erythromycin and clarithromycin (54%) when (7) isolates were resistant to each one. On the other hand, [illegible] of resistant to ß-lactam antibiotics used when (92%) of them were resistant to penicillin and ampicillin, followed by cloxicillin when (85%) of them were resistant to it, and (62%) of isolates were resistant to amoxicillin. Additionally (77%) of the isolates were resistant to tetracycline. Next to the previous two groups, comes metronidazole from nitromidazole group with (69%) percentage of resistance and amikacin from aminoglycoside group with the same percentage of resistance. Closed results were recorded by other investigator, Al-Baldawi (2001) found that resistant percentage to ß-lactam antibiotics were (100%). Results of the present study disagree with that by Al-Hadi (2001) who found that resistant percentage to ß-lactam antibiotics were (20%).

This could be explain by increasing colonization of the stomach with a mouth or intestinal flora that led to transfer antibiotic resistance - encoding plasmids to *H.pylori* from other bacteria (Kist *et al.*, 1997). Also resistance of ß-lactam antibiotics used may be due to possessing of ß-lactamase by the isolate which may be encoded by transferable plasmids and found in various *Enterobacteraceae* members, such as *E.coli*, *Proteus mirabilis*, *Klebsiella pneumonia* and *Salmonella typhimurium* (Vanzwert *et al.*, 1998; Gerits *et al.*, 2002). Or could be due to apparent of tolerant strains which have been identified by Dore *et al.*, (1999) who found that amoxicillin tolerance was detected among *H.pylori* strains after rescuer of amoxicillin resistance using gradient plate.

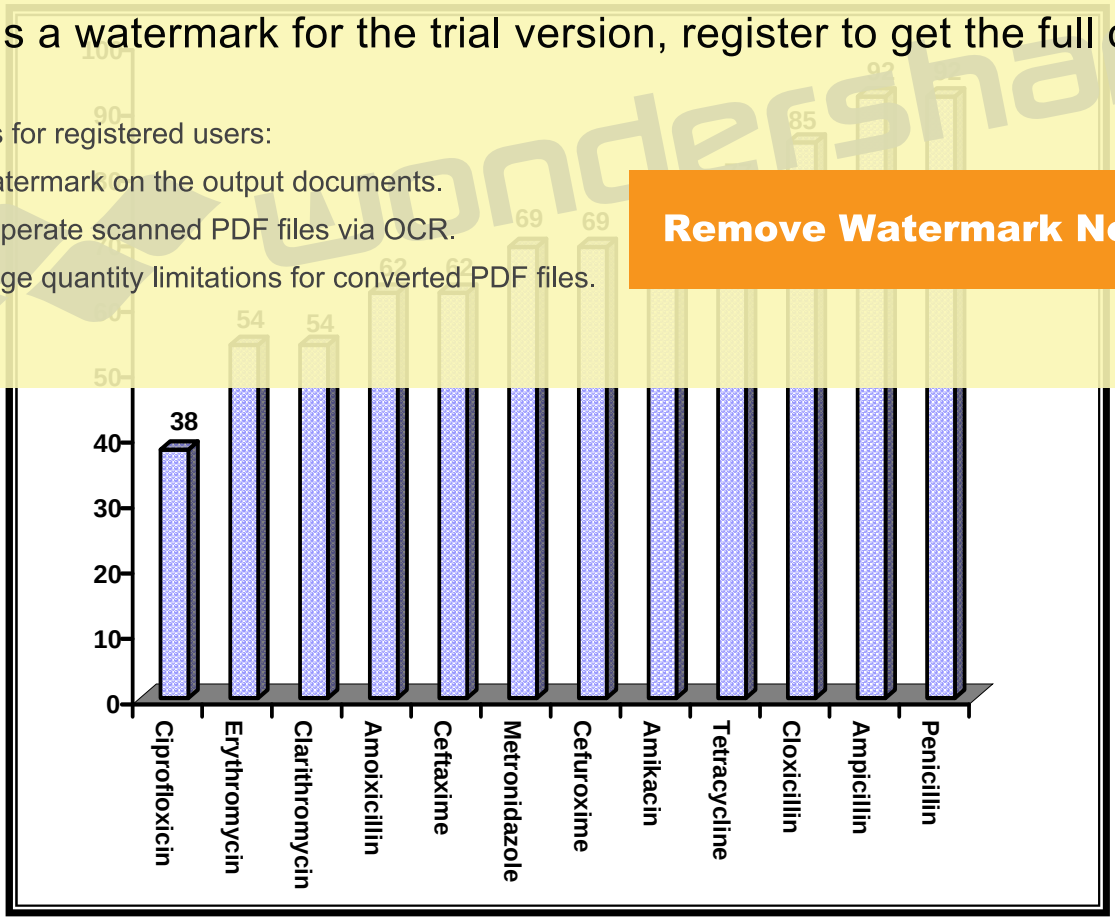


Figure (4-12): The Frequency of Resistance *H.pylori* Isolates.

Results also disagree with those obtained by Ossenkopp *et al.*, (2003) who reported that none of the *H.pylori* isolates were resistant to amoxicillin, and those obtained by Nahar *et al.*, (2004) in a study performed in Bangladesh who found that (6.6%) *H.pylori* isolates were resistant to amoxicillin. Amoxicillin resistance develops due to the structural alteration in one of the penicillin - binding protein (Delony and Schiller, 2000) or to the changes in other proteins involved in cell wall synthesis (Gerrits *et al.*, 2002).

This study also shows that metronidazole (nitromidazole group) possess relatively high resistant percentage (69%) of the isolates. Such results fall between those by Al-Hadi, (2001) who found it (70%) and Abdi *et al.*, (1999) with co-existed near stage of (59%). Almost similar results were obtained by Nahar *et al.*, (2004) who found that resistant percentage to metronidazole were (77.5%). recorded that [illegible] was sensitive to metronidazole, which means a (100%) resistant to such antibiotic.

The resistance mechanism of *H.pylori* to metronidazole is not well known. It may be related to the enzymes involved in reduction of the nitro group (Megraud *et al.*, 1999). Also Goodwin *et al.*, (1998) found that resistance to metronidazole may result from different alteration in the *rdx* A gene, which encodes an oxygen insensitive NADPH nitroreductase.

Rates of *H.pylori* resistance to metronidazole vary considerably. Some of this variation may reflect different techniques. Defining in vitro resistance, (54%) metronidazole resistance rate have been reported in Hong Kong compared with rates of around (40%) in the United

Kingdom and above (70%) in the developing countries. This high level of overall resistance obscures some important differences. Women are more likely to harbor resistance to *H.pylori* strains than men. Metronidazole also has been used for many years to treat gynecological infections in developing countries, and for diarrhoeal illness in the developing countries (Goodwin *et al.*, 1995). Present results also agree with those obtained by Ossenkopp *et al.*, (2003) who found that (80.9%) of *H.pylori* isolates were resistance to metronidazole.

High level of resistance to metronidazole may be due to frequent use for treatment of various infections (*Gardnerella* or *Trichomonas vaginitis*, ginidieosis, prophylaxis for hysterectomy) in patients with metronidazole resistant *H.pylori*. The patchy distribution of urease active antimicrobial over the gastric mucosa may cause wide variation in concentrations in the stomach, such [illegible] early lead to relapse or to develo[illegible] *et al.*, 2003; Nahar *et al.*, 2004).

Furthermore these results disagree with those obtained by Al-Hadi, (2001) and Al-Bldawi, (2001) who found that none of *H.pylori* tested isolates were resistant to clarithromycin, which mean that they were sensitive. Also the clarithromycin resistance rate was surprising in this study in which it was in contrary to results obtained by Trebesius *et al*., (2000) who reporting high rate of resistance to clarithromycin (100%). The explanation for development of macrolide resistance in *H.pylori*, particularly clarithromycin, is based on three defined mutations within 23S rRNA, resulting in decreasing of the antibiotic binding to the bacterial ribosome.

Versalovic *et al.*, (1997), on the other hand, reported that resistance to clarithomycin in clinical *H.pylori* isolates is caused predominately by distinct point mutations within the peptidyl transferase centre of 23S rRNA. While Megraud *et al.*, (1999) found that resistance to clarithomycin was found in 3% of *H.pylori* strains, due to detection of point mutation on the 23S rRNA gene. The rate by which antibiotic resistance emerges in a bacterial population within a patient will be determined by several factors, including rate of formation of the resistant mutant, biological cost of resistance and the rate and pattern of antibiotic use (Lipsitch and Levin, 1997) so, from results reported and shown in (Table 4-13) isolates HP1, HP2, HP3, HP5 and HP6 were the most resistant isolates to amoxi

4.1.1.8 Minimum Inhibitory Concentration (MIC) of Antimicrobial Agents Against *H.pylori*:

In the last ten years, eradication of *Helicabacter pylori* has been the subject of numerous clinical traits in order to find the optimum therapy. There are several limitations to previous studies of antimicrobial characteristics of *H.pylori* strains, which include small size, no details about the history of the patients, a geographical distribution limited to specific areas, and inadequate methodology, with few exceptions (McMahon *et al*., 2003).

The MICs of amoxicillin, penicillin, metronidazole, tetracycline and clarithromycin for the highly rate resistant *H.pylori* isolates were confirmed by agar dilution MIC measurement and are shown in (Figure 4-13 A, B, C, D and E). High - level of amoxicillin - resistance *H.pylori* (HP2, HP6) isolates (MIC, 512µg/ml) was observed. Followed by two *H.pylori* (HP1, HP5) isolates with a moderate - level of resistant (256µg/ml) and one isolate HP3 with a low rate resistant to amoxicillin (128µg/ml). Two *H.pylori* (HP1, HP5) isolates showed high level penicillin resistance with MIC of (512µg/ml) and two (HP3, HP6) isolates with a moderate level resistance to penicillin (256µg/ml). While one (HP2) isolate showed a low resistant (128µg/ml). On the other hand, one isolate (HP3) showed a high level of resistance to metronidazole (512µg/ml) followed by (HP2) with (256µg/ml) while HP1, HP5 isolates showed moderate resistance (128µg/ml) and one isolate HP6 (32µg/ml), as well as HP1 isolate of high level of resistant to tetracycline with an MIC value of (256µg/ml), followed by HP6 isolate (128µg/ml), while HP2, HP5 isolates showed low levels of resistant to tetracycline with MICs of (64µg/ml) for each. While HP3 isolate was sensitive to this antibiotic (32µg/ml), HP3 and HP6 isolates possessed moderate resistant to clarithromycin (128µg/ml) for each, and HP1 with slightly level of resistant (64µg/ml), as well as (HP2, HP5) isolates (32µg/ml) for each.

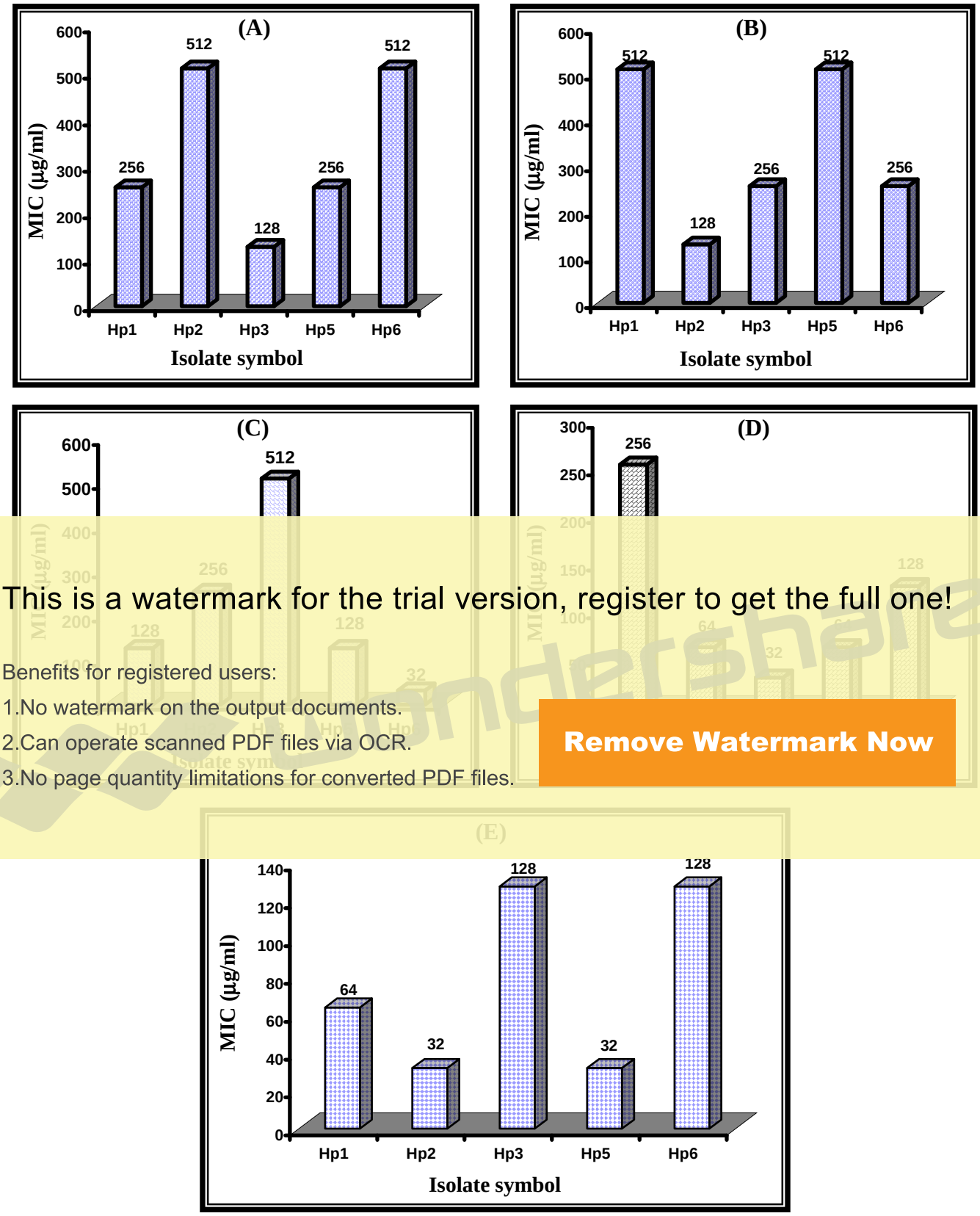


Figure (4-13): Minimum Inhibitory Concentrations (MICs) Against *Helicobacter pylori* Isolates.
A- Amoxicillin. B- Penicillin.
C- Metronidazole. D- Tetracycline.
E- Clarithromycin.

Results of present study were in agreement with those obtained by Dore *et al.*, (1999) when MIC for all *H.pylori* strains were more than (256mg/L) due to the tolerance to ß-lactam antibiotics phenomenon. They added that amoxicillin resistance was lost after storage of strain at (-80°C) (but rescued by plating these strains on to amoxicillin gradient plates). *H.pylori* strains were rescued up to amoxicillin resistance to (64) and (128mg/L).

Several studies have shown that penicillin - tolerant bacterial strains such as group A S*treptococci*, may lose their penicillin resistance phenotype after storage, and such resistance could be restored by consecutive transfer to penicillin of the gradient agar plate method, the amoxicillin resistance phenotype of *H.pylori* could rescued (Sabath *et al.*, 1977).

Highest rate of MIC distribution for amoxicillin against *H.pylori* was [illegible] modification in penicillin binding proteins or to the presence of beta-lactamase (Goodwin *et al.*, 1989). Similar results are recorded by Piccolomini *et al.*, (1997) who tested (71) isolates of *H.pylori* and found that the MICs of (90%) of the isolates were inhibited by agar dilution of (1 mg/L) for clarithromycin.

Haredy *et al.*, (1988) found that clarithomycin with an MIC of (0.03mg/L) was the most active among macrolides of those being tested.

The MIC distribution for clarithromycin against *H.pylori* was within normal value. Although some isolates showed lower or moderate resistance, but this could be due to a point mutation on the 23S rRNA gene (Hirschl *et al.*, 2000). This may reflect the limited use of macrolides in Iraq. Another explanation may be that isolates obtained from patients with duodenum ulcer are more likely to be susceptible to macrolides, also diffusion of drug in the gastric lumen decreases when the pH increases (Chisholm and Owen, 2004).

A recent study done in India by Mukhopadhyay *et al*., (2000) show that none of *H.pylori* strains were resistant to clarithromycin, which in accord with macrolides not being used very often in India. In contrast, resemble results to the present declared that (90%) of these isolates were resistant to at least (8 µg/ml) MIC concentration of metronidazole. These data indicate that frequency of metronidazole resistance *H.pylori* is extremely high in India. It was found, in India that metronidazole resistant results from mutation of the chromosomal *rdx* A nitroreductase gene, not from the acquisition of new "resistance gene" (e.g. in plasmids or transposons). Another investigator Sisson *et al.* (2000) indicated that metronidazole is a mutagenic agent due to the frequent use of it against variety of illness. Generally such doses may induce and select for resistant mutants of resident *H.pylori* strains without eradicating the infection. Alberton *et al.* (1995) found that some bacteria escape treatment at sanctuary sites from which they emerge after the stop of

The resistance mechanism of *H.pylori* to metronidazole is not well known. It may be related to the enzymes involved in the reduction of the nitro group, but alternate pathways may exist, therefore, observed MIC would be the result of a complex phenomenon (Kato *et al*., 2000).

The break point of (8 µg/ml) has been proposed for metronidazole resistance based on studies using bismuth based triple therapies (Lui *et al*., 2003). Megraud *et al.*, (1999) found that distribution of the MICs of clarithromycin, metronidazole and amoxicillin determined by agar diffusion method were only (14%) of strains showed intermediate or resistant to clarithromycin, while (27%) of strains resistant to metronidazole. Such resistance was probably due to technical problems related to the method metronidazole testing. It is commonly accepted that

emergence of primary resistance and acquired resistance to metronidazole in *H.pylori* is usually associated to the treatment failure. Differences between the resistance rates may reflect the variation in metronidazole usage between countries since metronidazole alone can easily induce resistance to the drug in *H.pylori*. Furthermore, methological variables and differences in interpretation of susceptible test results may also contribute to the varied resistance rates (Alarcon *et al*., 1999).

One of the prominent reasons for treatment failures is that *H.pylori* strains that survive after eradication therapy remain in the surface mucous gel layer is more frequent than on the surface of mucous cells (Shimizu *et al*., 1996). This finding may indicate that concentrations of the drugs administered cannot fully eradicate *H.pylori* in the mucous gel layer. Drug concentrations in the mucous gel layer itself have not been determined. Another reason is the standardization of the MIC corresponding to the cut off level determine the break point resistance. The results of our study were in agreement by Kobayashi *et al*., (2004) who found that the MIC values of *H.pylori* isolates against clarithomycin and amoxicillin were (32µg/ml) and (1 µg/ml). Similar results were also found in Germany by Han *et al*., (1999) when high levels amoxicillin resistance (MIC > 265 µg/ml) were reported.

Ossenkopp *et al*., (1995) indicated that MIC values are greatly affected by the environmental conditions including the CO_2 concentration. MIC values of macrolide antibiotics for *H.pylori* isolates in particular are significantly affected by presence of CO_2 because of acidification of the test medium. NCCLs, (1999) established that the MIC interpretive standard of clarithromycin for *H.pylori* is defined as: susceptible (≤ 0.25 µg/ml), intermediately resistant, (0.5 µg/ml) and resistant (≥ 1 µg/ml).

The rate by which the resistant mutant forms will be determined by mutation rate and population size. Mutating bacteria might be important because their higher mutation rate may enhance the supply of resistance mutations. After the appearance of resistant mutants, their spread and maintenance will be influenced by the rate and pattern of antibiotic use (selective pressure) and effect of the particular resistance on bacterial. Byörkholm *et al*., (2001) found that the mutation frequency in *H.pylori* indicates that hypermutable strains are common and those strains exhibite a wide range of mutation frequencies. In addition, results showed that the high fitness cost was associated with clarithromycin resistance mutation which was reduced in some clinical isolates.

Most *H.pylori* strains tested show higher mutation frequencies than the wild type *Enterobacteriaceae*, and one - fourth of the strains showed mutation frequencies higher [illegible] (Gil[illegible], 1998; Horst and Mazi[illegible], 1999) [illegible] all bacteria in the stomach, except few that are resistant, die. The resistant bacteria grow slowly, and during growth a new variant grown with a faster growth rate than the original resistant mutant (Byörkholm *et al*., 2001).

Results also agreed with those obtained by Owen, (2002) who found that the MIC values of *H.pylori* isolates against clarithromycin were (> 2µg/ml), which is due to variant point mutations in the peptidyl transrase region of domain V of the 23S rRNA gene, *H.pylori* has two copies of that gene, and the mechanism of resistance to clarithromycin appears to be decreased ribosome binding of the macrolide so that it fails to act by interrupting protein biosynthesis.

4.1.1.9 Inhibitory Activity of *Lactobacillus acidophilus* on *H.pylori*:

Current antibiotic treatment for *H.pylori* infection is often associated with frequent adverse effects and resistance to antibiotics. Alternative methods to control *H.pylori* infection are needed.

Some specific strains of lactic acid bacteria (probiotics) in dairy products are known to inhibit growth of *H.pylori* in vitro (Wondakoon *et al.*, 2002).

Numerous studies on human suggest that lactic acid bacteria at a level of $(10^{9}$-$10^{11})$ per day can decrease the incident, duration and severity of some gastric and intestinal illness. In the present study, inhibitory effect of *L.acidophilus* on *H.pylori* was investigated using solid and liquid media. Result of this part of the study could be described as follows:

4.1.1.9.1 on Solid Media:-

Ability of the LAB isolates to produce inhibition activity on pathogenic bacteria was tested by growing the isolate on MRS agar medium. In this approach, AL- Khafaji (1992) mentioned that using MRS agar medium in studying the ability of LAB isolates to produce inhibiting materials when grown under anaerobic condition is the choosing procedure that gives reasonable results.

(Table 4-12) shows the inhibitory activity of *Lactobacillus acidophilus* isolate grown on MRS agar against three highly resistant *H.pylori* isolates at three different incubation periods.

Table (4-12): Inhibitory Effect of LAB against *H.pylori* Isolates on Solid and in Liquid Media Estimated by Diameter of Inhibition Zone (mm).

Diameter of Inhibition Zone						
on Solid Media				in Liquid Media		
Incubation Periods						
Isolate	24hr	48hr	72hr	24hr	48hr	72hr
HP1	-	5	7	10	23	23
HP2	-	5	5	10	22	25
HP3	5	7	7	15	17	17

Results show that *L.acidophilus* exhibited moderate inhibitory effect against *H.pylori* isolates on solid media when the inhibitory zone reached 5mm after (24hr) with HP3 isolate, while no effect was detected on HP1 and HP2 isolates during this period. Furthermore, after (48hr) of incubation there was a slight increase in the inhibition zone observed when both HP1, HP2 isolates reached to (5mm) and (7mm), In contrast, after (72hr) no inhibitory effects were observed against HP3 and HP2 isolates, while a slight increase was reported in the inhibition zone of HP1 which reached to (7mm) (Figure 4-14).

Garver and Muriana (1994) mentioned that production of inhibited materials by LAB is dependent on the medium used for growth, and they found also that tween (80) induced the production of protein (bacterocin) by increasing activity of the bacteria. This result almost agreed with those obtained by Al-Dulemy (2000) who found that the inhibitory effect of LAB increased after (48 hr) of incubation. But Al-Obidy (1997) and Al-Jeboury (2005) found that LAB gave inhibitory effect after (24hr) Differences in the above results of LAB against the pathogenic bacteria may be related to the type of bacteria, type of inhibitory substance, its quantity and ability to distribute in the medium (Egoroy, 1985).

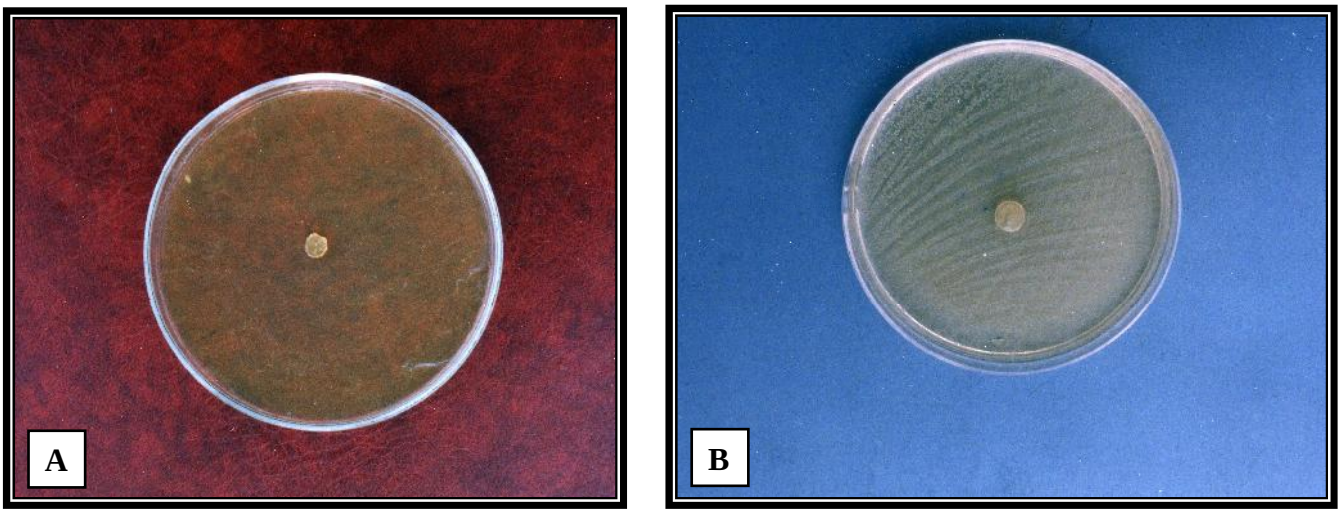


Figure (4-14): Inhibitory Effect of *L.acidophilus* Isolate Against *H.pylori* Isolates (HP1, HP2 and HP3) on Solid Media.

A- *L.acidophilus* Inhibitory Effect on HP2 Isolate After (48hr).

B- *L.acidophilus* Inhibitory Effect on HP3 Isolate After (48hr).

C- *L.acidophilus* Inhibitory Effect on HP1 Isolate After (72hr).

Garver and Muriana (1994) mentioned that production of inhibited materials by LAB is dependent on the medium used for growth, and they found also that tween (80) induced the production of protein (bacterocin) by increasing activity of the bacteria. This result almost agreed with those obtained by Al-Dulemy (2000) who found that the inhibitory effect of LAB increased after (48hr) of incubation. But Al-Obidy (1997) and Al-Jeboury (2005) found that LAB gave inhibitory effect after (24hr) Differences in the above results of LAB against the pathogenic bacteria may be related to the type of bacteria, type of inhibitory substance, its quantity and ability to distribute in the medium (Egoroy, 1985).

inhibitory effect is correlated to the concentration of lactic acid produced by LAB examined.

4.1.1.9.2 in Liquid Media:-

Well diffusion method was used to determine the inhibition activity of *L.acidophilus* filtrates, grown at different incubation periods (24hr, 48hr and 72hr) against three *H.pylori* isolates. By filling the wells with Brain - heart infusion agar, plates have been cultured by HP1, HP2 and HP3 isolates with the filtrates of LAB isolates.

Maximum inhibition zone diameters reached (20mm) which is highest than that recorded by the solid media. This may be due to the ability of MRS broth to exhibit wide spectrum inhibitory effect against Gram - positive bacteria (*S.aureus*, *Bacilus subtilis*) and Gram - negative bacteria (*E.coli*, *Klebsilla* spp., *Proteus* spp.) when inhibition zone diameter ranged between (13-19)mm. (Gupta *et al.*,1998).

(Table 4-12) exhibits the inhibitory effect of *L.acidophilus* filtrates. Results of (24hr) period of incubation showed best inhibitory effect when inhibition zone diameter reached to (10mm) against tested HP1, HP2 isolates, and to (15mm) against HP3 isolate. While increasing incubation period to (48hr) showed highest inhibitory effect when the inhibition zone increased (1[illegible] against HP3 isolate and to (20mm), (23mm) for both HP2 and HP3 isolates, respectively.

[illegible] increasing incubation period to (72hr) showed highest effect for LAB filtrate upon HP1 and HP3 except HP2 which exhibited highest inhibition zone (25mm) after this period of incubation (Figures 4-15 and 4-16).

Results disagreed with those obtained by Al-Jeboury, (2005) who found that best inhibitory effect was obtained after (24hr) of incubation with (18mm) inhibition zone diameter, and added that increasing incubation period to (48hr) resulted in least inhibitory effect for LAB isolate. The reason for such result may be that the inhibitory materials (acidophilin, plantaracin) are secreted outside the cells after increasing the incubation time causing decrease in the inhibitory activity.

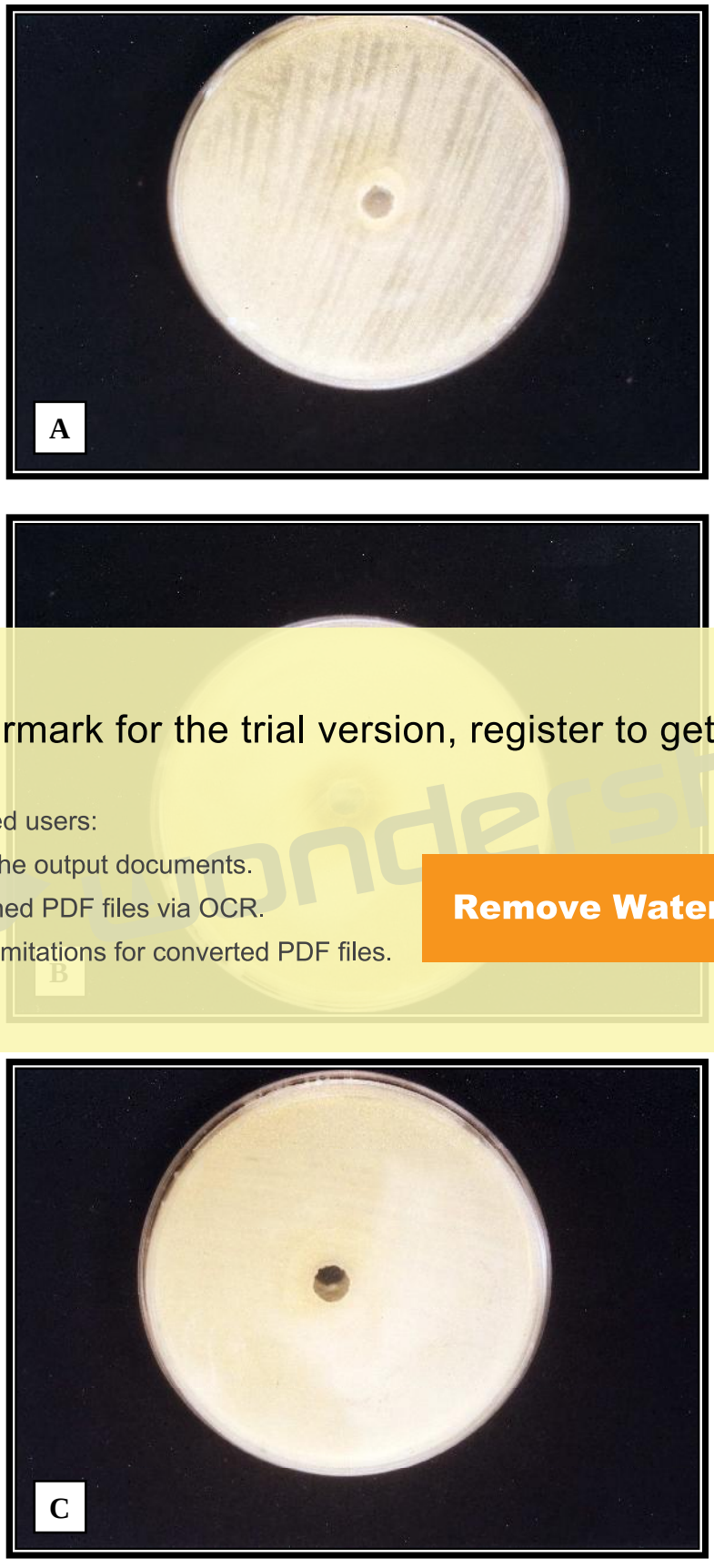


Figure (4-15): Inhibitory Effect of *L.acidophilus* Against (A-HP1, B- HP2 and C- HP3) Isolates) in Liquid Mdia (MRS Broth) after (42hr).

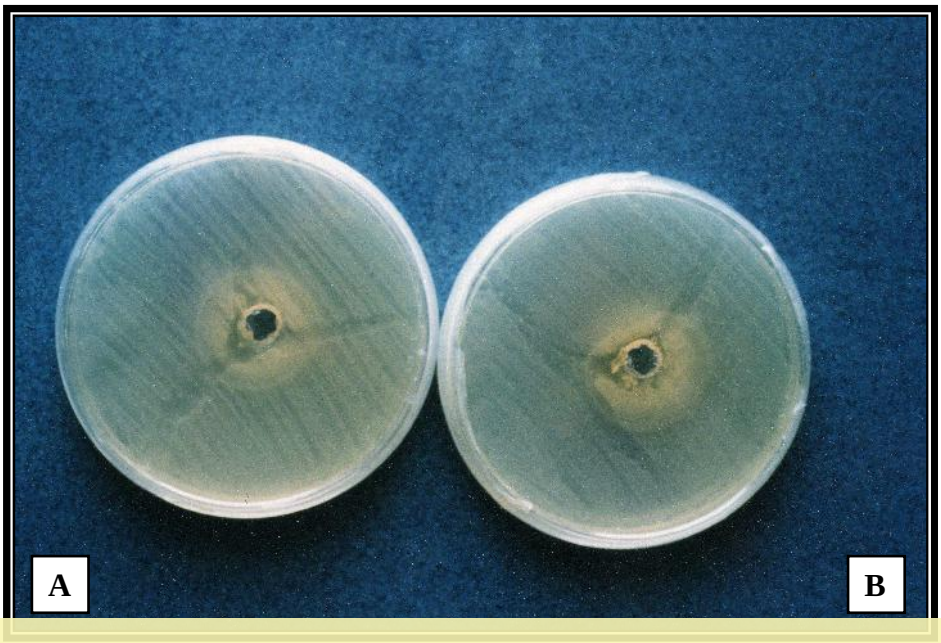


Figure (4-16): Inhibitory Effect of *L.acidophilus* Against *H. pylori* (A-HP1, B- HP2 and C-HP3) Isolates in Liquid Medium (MRS broth) After (72hr).

Several studies found that incubation time of (18hr) and (48hr) gave less inhibitory effect than that effect after (24hr) incubation. Pfeiffer and Radler (1982) found a relationship between the diameter of inhibition zone and concentration of the inhibitory substance. On the other hand, Barfoot and Klaenhammer (1983) declared that death of tested bacteria increased with increasing inhibitory substances like bacteriocin, acidophilin and plantaracin of LAB. Furthermore similar results were obtained by Al-Dulemy, (2005) who found that the inhibitory effect increased after (48 hr) probiotic strains may inhibit pathogenic bacteria both in vitro and in vivo through several different mechanisms. Production of directly inhibitory compounds (e.g., bacteriocin), reduction of luminal pH through short chain fatty acid production, competition for nutrients, adhesion sites on the gut [illegible] expression are some of these mechanisms (Fooks and Gibson, 2002). Effects of LAB on *Helicobacter* infection in humans have promising [illegible] *H.pylori* were [illegible] (Michetti *et al.*, 1999). Sakamoto *et al.*, (2001) indicated that LAB reduced [C^{13}] urea breath test values, and therapy with *L.acidophilus* was shown to reduce gastric mucosal inflammation. Sgouras *et al.*,(2004) reported potential inhibitory effect of *Lactobacillus casei* strain on *H.pylori* by using in vitro inhibition assays and in vivo with mouse model, they found that in vitro activity against *H.pylori* was observed in presence of viable *L.casei* strain cells but not in the cell - free culture supernatant. Wang *et al.*, (2004) found that *Bifidobacterium* (Bb12) exerted an in vitro inhibitory effect against *H.pylori*, whereas *L.acidophilus* showed no such effect. (Table 4-13) shows the inhibitory effect of concentrated filtrate of *L.acidophilus* against three *H.pylori* isolates. Filtrate of *L.acidophilus* was concentrated to four folds by freeze - dryer. The one - fold concentrated filtrate of LAB gave inhibition zone diameters of (7, 8 and 9) mm against HP1, HP2 and HP3

isolates, respectively. Two - fold filtrate showed noticeable inhibitory effects with zone diameters of (10, 12 and 15) mm against HP3, HP1 and HP2 isolates, respectively. While the three and four - fold filtrates exhibited the highest inhibitory effects after (24hr) incubation. Diameter of three - fold of *L.acidophilus* against HP3, HP1 and HP2 isolates reached to (15, 20 and 23) mm, respectively ,while the four - fold one reached to (23 and 25) mm) for both of HP3 and HP1, respectively. As seen in (Figure 4-17).

Table (4-13): Effect of Non Concentrated and Concentrated LAB Filtrates.

Isolate No.	Inhibition zone diameter (mm)				
	LAB filtrate				
	Non concentrated		Concentrated		
	Control (Broth)	One -fold	Two -fold	Three -fold	Four -fold
HP2	7	8	15	23	-
HP3	7	9	10	15	23

Coconnier *et al.*, (1998) reported that conditional media from *L.acidophilus* reduced the viability of *H.pylori* in vitro, independent of lactic acid concentrations.

Several in vitro studies were conducted to ascertain whether the effects of LAB on *H.pylori* survival and function are due to lactic acid or to other antibacterial products generated by LAB such as bacterocin. Of the several bacteriocin tested, lacticins produced by several *Lactobacillus* species were shown to have the greatest anti *Helicobacter* activity when used against several strains of *H.pylori* (Kin *et al.*, 2003).

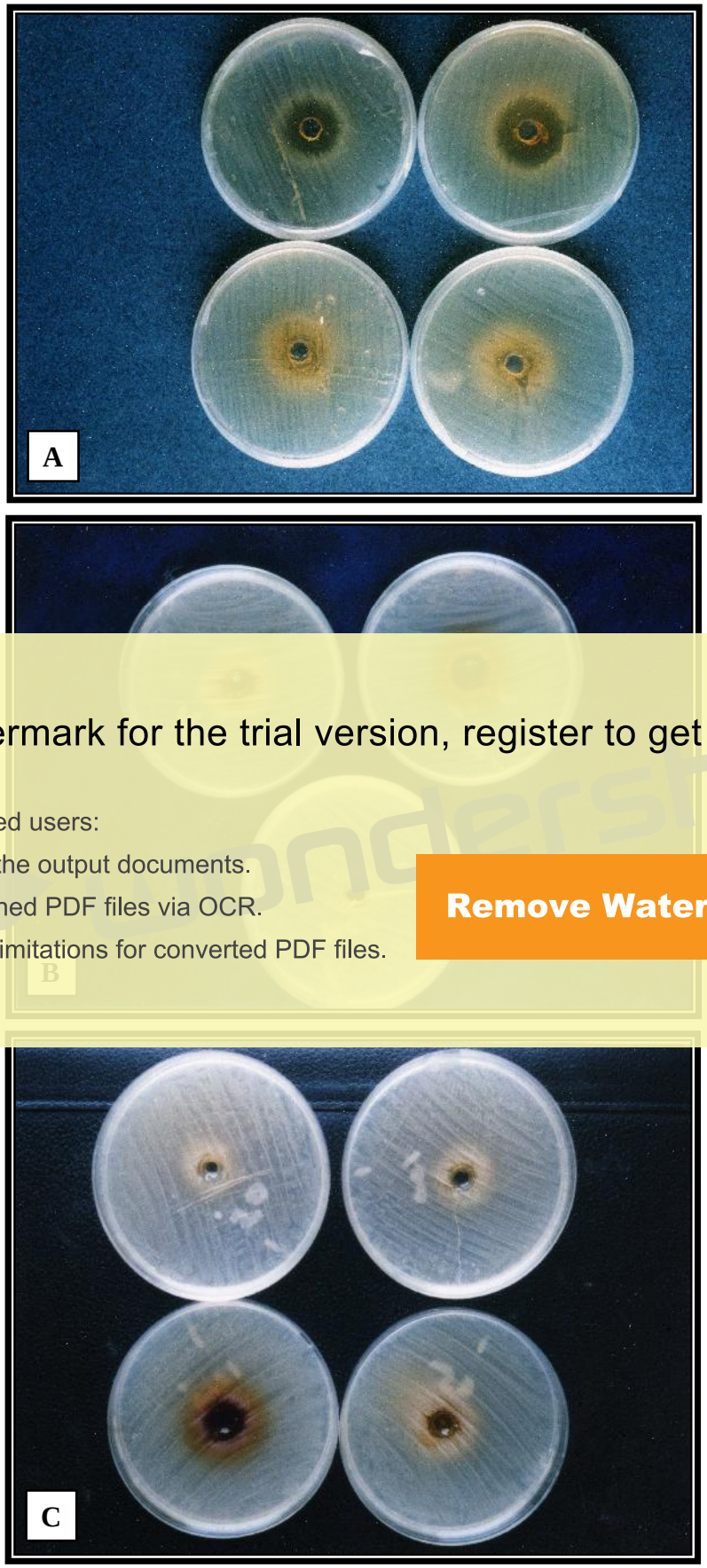


Figure (4-17): Inhibitory Effect of Concentrated Filtrate of *L.acidophilus* Against *Helicobacter pylori* (A-HP1, B-HP3 and C-HP2) Isolates.

4.1.1.10 Minimum Inhibitory Concentration (MIC) of LAB Filtrates Against *H. pylori*:

Many factors influence MIC estimation, inoculums size, pH, temperature and nature of cell wall (Nikaido, 1989).

To determine MICs of the LAB filtrates required to inhibit or minimize adhesion property of *Helicobacter pylori*, serial dilutions were prepared from the four- fold filtrates of *L.acidophilus* isolates as previously mentioned (3.2.13.3). (Table 4-14) declares that the first two concentrations (1:9 and 2:8) had no observed effect against HP1 and HP2 isolates when heavy growth of these bacteria were noticed after (24hr) of incubation, while HP3 isolate showed decrease in growth to the medium level at these concentrations.

Table (4-14): Minimum Inhibitory Concentrations (ug/ml) of LAB Filtrates against *H.pylori* Isolates.

Strains	Concentrations Filtrates									
	1:9	2:8	3:7	4:6	5:5	6:4	7:3	8:2	9:1	10:0
HP1	+++	+++	+++	++	++	+	-	-	-	-
HP2	+++	+++	+++	++	+	-	-	-	-	-
HP3	+++	+++	++	+	+	-	-	-	-	-

Heavy Growth = +++ Medium Growth = ++ Light Growth = + No Growth= -

Coming next, concentrations (3:7 and 4:6) of HP1 isolate which caused decrease in growth level to medium, but the growth of HP2 isolate decreased to the medium level at concentrations (3:7). Sharp decrease in growth to light level was recorded by HP2 and HP3 isolates at concentrations (3:7 and 4:6).

Growth of *H.pylori* isolates were sharply decreased through a light growth level at concentrations (4:6 and 5:5) with HP2, HP3 and HP1, respectively. With concentration (5:5), the situation was different when no

growth was observed for HP2 and HP3 isolates, while light growth was observed with HP1 isolate. The last three concentrations of *L.acidophilus* (6:4, 7:3, 8:2) were quite enough to retard any growth of *H.pylori* isolates relating to the just mentioned finding. It may be concluded that filtrate concentration of (5:5) is the MIC for HP2 and HP3 isolates of *H.pylori* and (6:4) concentration for HP1 isolate of *H.pylori.*

Similar results were recorded by Al-Jeboury (2005) who found that the MIC of *L.acidophilus* concentrated filtrates were (50%) and (60%), which are completely inhibited the growth of *P.mirabilis* isolates. Furthermore, Abideen, (2005) found that the MIC required to retard any growth of *P.mirabilis* was at (5:5) and (6:4) concentrations.

4.1.1.11 Adhesion of *Helicobacter pylori*:

In vitro adherence of *H.pylori* to urothelial is considered as an important virulence factor in pathogenesis [illegible] adherence property of *H.pylori* as may be affected by LAB isolate, was investigated.

Adherence ability of *H.pylori* to healthy and infected ureoepithelium (UEP) observed under oil immersion objective of the compound light microscope is shown in (Figure 4-18). HP1, HP2 and HP3 appeared as curved rod or small coccoid shape adhered to the ureoepithelium. Average number of bacteria adhered to each UEP cell were (60 to 75), (70 to 85) and (80 to 95) for HP1, HP2 and HP3 isolates, respectively. Such results were near those recorded by each of Smoot *et al.*, (1999) who found that highest number of adherent *H.pylori* were 100 bacterial / gastric epithelial cell and Al-Jeboury (2005) when (55) of *P.mirabilis* were adhered to each UEP cell.

In antral and duodenal biopsy specimens, *H.pylori* has been shown to be attached to the epithelial cells and occasionally penetrate the cells.

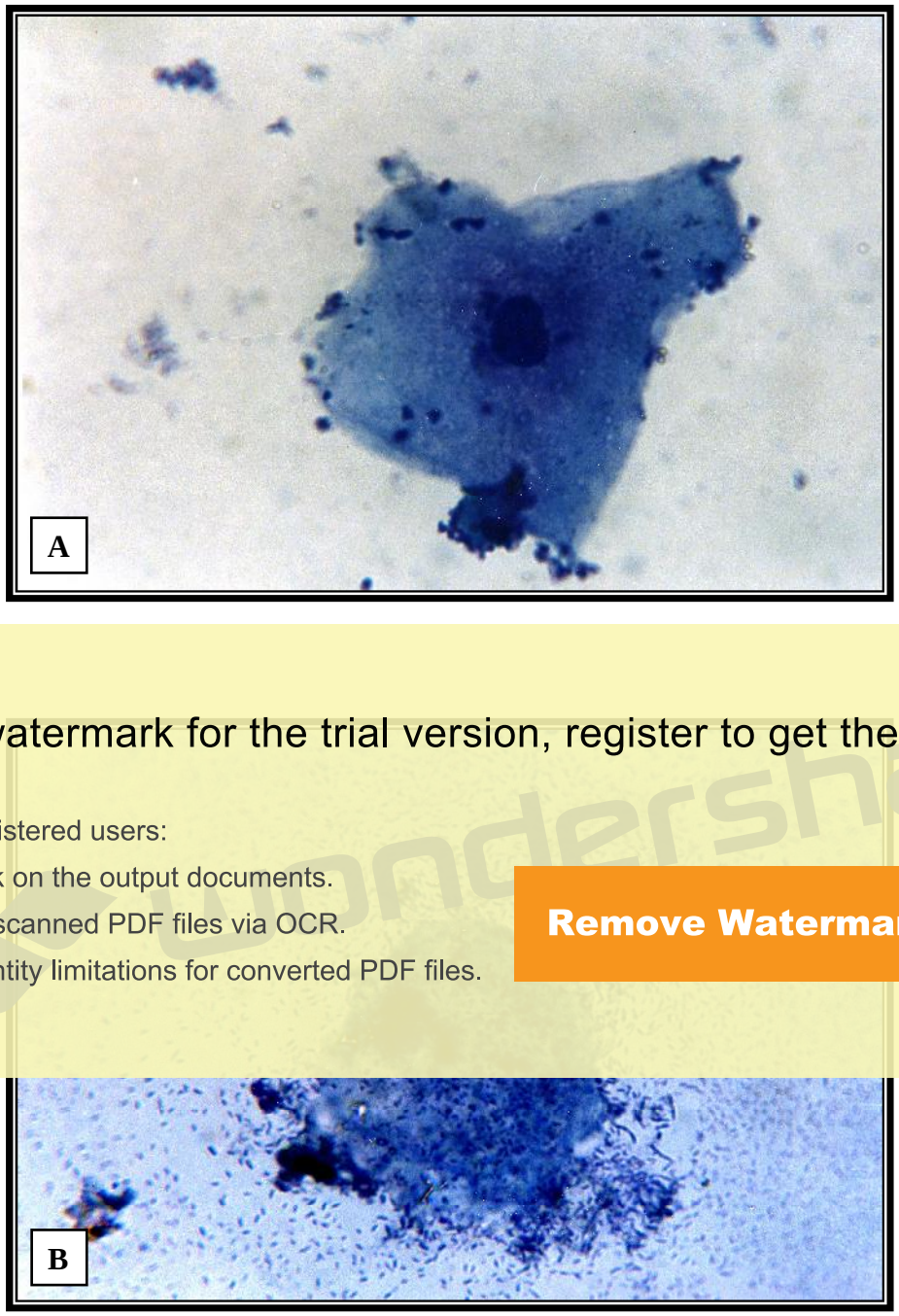


Figure (4-18): Microscopical Examination of Adhesion Property of *Helicobacter pylori* (HP1) Isolate to Ureoepithelium Cell under Oil - Immersion Objective (100xs).

A- Normal Uroepithelial Cell

B- *Helicobacter pylori* (HP1) Isolate Adhered to Ureoepithelial Cell.

4.1.1.11.1 Adhesion Inhibition by LAB Filtrates:-

Adherence to the gastric mucosa may play an important role in the colonization and pathogencity of *H.pylori*. Approximately one fifth of organisms are adherent to the gastric mucosal surface, whereas the remaining appear to be free - living within the mucus layer (Lee *et al.*, 1993).

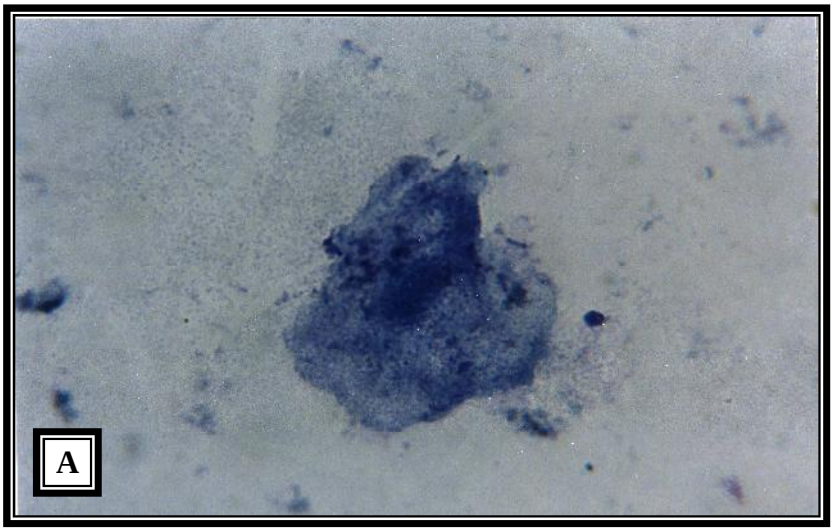
When potential inhibitory effect of the concentrate filtrates of *L.acidophilius* against adhesion property of *Helicobacter pylori* isolates were studied, results showed that the four- fold concentrated filtrate of *L.acidophilus* was able to minimize adhesion of HP1,HP2 and HP3 isolates to the uroepithelial cells reaching an average of (5-15) bacteria / cells as shown in (Figure 4-19). It was observed that adhesion of HP1, HP2 and HP3 isolates to UEPCs were clearly minimized. This may be due to the effect of inhibitory substances found in the filtrate of LAB isolate.

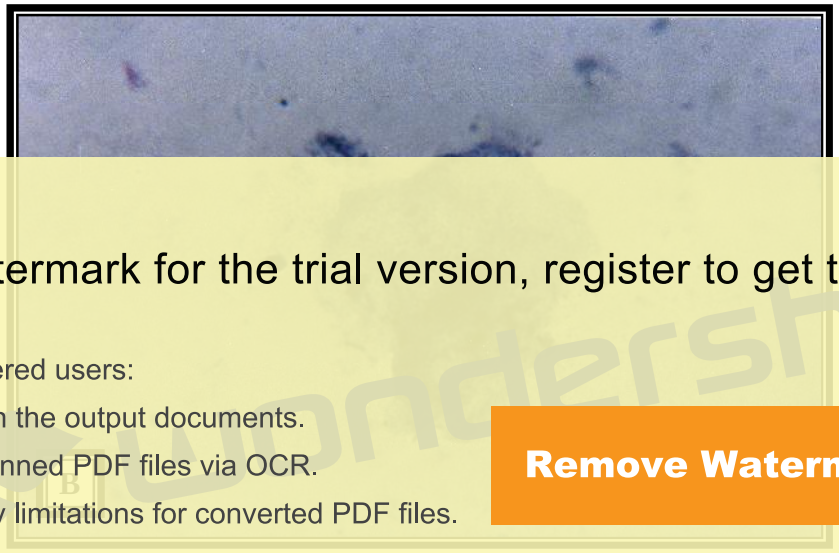
Kaur *et al.*, (1997) found that [illegible] effect of the attachment of *H* [illegible] lines. Wendakoon *et al.*, (2002) examined the efficacy of a specially designed fermented milk product containing selected lactic acid bacteria with anti-*Helicobacter pylori* properties (including *L.casei*, *L.acidophilus*, and *S.thermphilus)*. After administration of the yoghurt for one month, only one subject of 27 was found to have a complete eradication. Similar results were recorded by Michetti *et al.*, (1999) who found that specific strains of *L.acidophilus* are known to inhibit intestinal cell adhesion and invasion by enterovirulent bacteria. As *L.acidophilus* is able to survive transiently in the human stomach, it may reduce *H.pylori* infection. *L.acidophilus* supernatant inhibited *H.pylori* growth in vitro, also marked decrease in breath test values were observed immediately after treatment with *Lactobacillus acidophilus* culture supernatant.

Ability of LAB to decrease the gastrointestinal invasion of pathogenic bacteria has also been described. Bernet *et al.,* (1994) reported a dose - dependent *L.acidophilus* mediated inhibition of the adherence of enteropathogenic *E.coli* and *Salmonella typhimurium* to the enterocyte cell-line Caco-2. In addition, *L.acidophilus* inhibited the entry of *E.coli, S.typhimurium* and *Yersinia pseudotuberculosis* into Caco2-2 cells. Ability of LAB to compete with pathogens for adhesion to the intestinal wall is influenced by their membrane fluidity. This possibility was suggested by studies indicating that the type and quantities of polyunsaturated fatty acids in the extracellular milieus influence the adhesive properties of LAB to the epithelium (Kankaanpaa *et al.,* 2001; Kankaanpaa *et al.,* 2004)

Lactobacilli are the predominant bacteria found in normal fasting [illegible] is small ra[illegible] *L.acidophilus* is a strain of human origin that can tolerate very low acidic pH and can survive passage through the gastrointestinal tract. Being acid resistant, *L.acidophilus* persists in the stomach longer than other bacteria (Conway *et al.,* 1987).

Probiotics may protect the host from intestinal disorders by inhibiting colonization by other pathogenic strains (considered to be colonization resistance). For example, competitive inhibition for bacterial adhesion sites on intestinal epithelial surfaces is one of several modes of action of probiotic (Kleeman and Klaenhammer *et al.,* 1982; Golden *et al.,* 1992). Some probiotic strains have been chosen for their ability to adhere to epithelial cells (Rolfe, 2000).





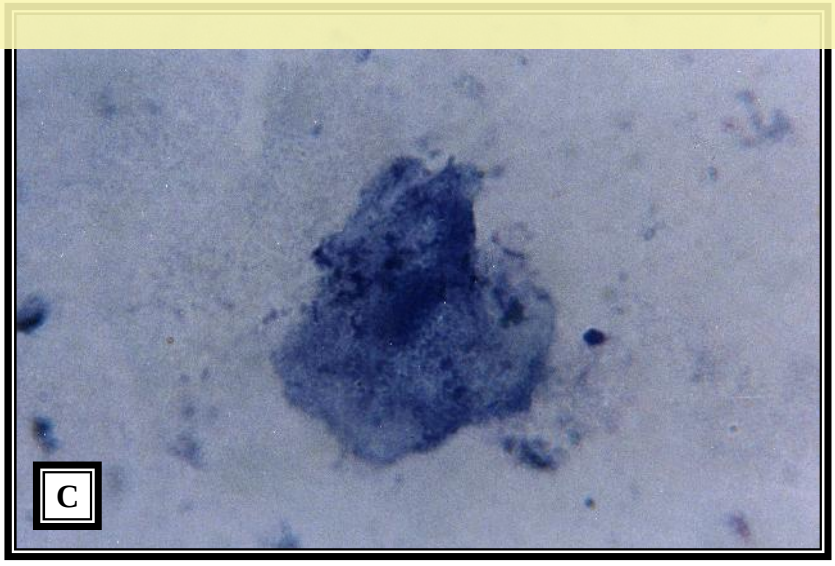


Figure (4-19): Microscopical Examination Adherence of *H. pylori* (A- HP1 B- HP2 and C–HP3) Isolates to The Uroepithelium Cells After Treatment with Concentrated Filtrate of LAB (1000 X).

Upon concentrated filtrates of LAB treatment, *H pylori* HP1, HP2 and HP3 isolates showed sequence of morphological changes, the first sign of those was the formation of precoccoid form that altered to the coccoid form of LAB treated *H.pylori* isolates. As shown in (Figure 4-20). Transformation of *H.pylori* into coccoid forms explain its survival in unfavorable conditions, *H.pylori* has a helical bacillary appearance in the favorable conditions which undergoes transformation (Caternich and Makin, 1991; Cellini *et al.*, 1994).

Several authors have proposed that coccoid forms are a mechanism by which *H.pylori* can survive harsh environmental condition, or is able to convert to resistant forms under conditions of [illegible] believe that the coccoid forms of *H.pylori* are the morphological manifestation of bacterial [illegible] activity of *H.pylori* was examined, it was found that the urease activity of HP2, HP3 and HP1 rapid urease producing isolates decreased after treatment with LAB concentrated filtrates when positive results for color change were obtained after (10, 18 and 24) hr, respectively.

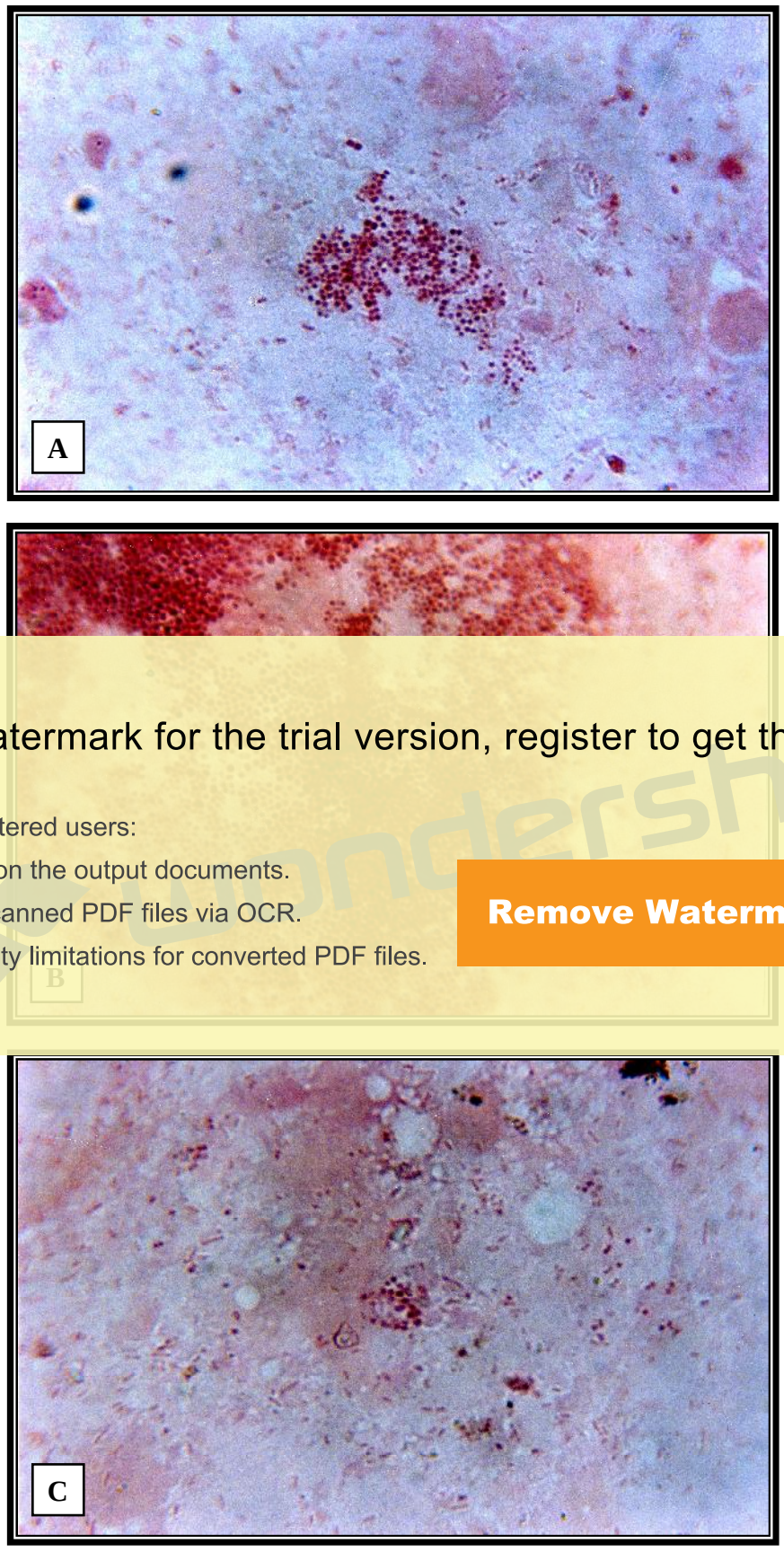


Figure (4-20): The Coccoid Forms of *H. pylori* (A- HP1, B- HP2 And C- HP3) Isolates after Treatment with Concentrated Filtrate of LAB.

This was due to the effect of lactic acid and other inhibitory substances found in the concentrated filtrate of LAB that inhibited *H.pylori* urease activity.

Sqouras *et al.*, (2004) studied the potential inhibitory effect of *L.casei* strain on *H.pylori* and observed that in the presence of viable *L.casei*, but not in cell free culture supernatant, the urease activity of *H.pylori* was inhibited. Also significant reduction in the levels of *H.pylori* colonization was observed in the antrum and body mucosa in vivo in the *Lactobacillus* treated study group. This reduction was accompanied by significant decline the chronic and active gastric mucosal inflammation. Similar results were obtained by Coconnier *et al.*, (1998) which found that LAB - spent culture supernatant treatment inhibited the *H.pylori* urease activity in vitro and in *H.pylori* that cells. Also infection by *H.felis* decrease after treatment with LAB-SCS.

While McGowan *et al.*(1995) reported a dramatic decreased in [illegible] at pH 2, and [illegible] enhanced in presence of urea resulting from urease activity of *H.pylori*. Michetti *et al.*, (1999), found that culture supernatant of *Lactobacillus* showed marked decrease in C^{13}- urea breath test values in humans.

Felley *et al.*, (2001) demonstrated that a 3-week intake of acidified milk containing *L.johnsonii* decreased *H.pylori* density in humans. Horie *et al.*,(2004) designed a specially functional drinking yogurt containing *L.acidophilus* and *Befidobacterium* species with (1%) egg yolk. Immunoglobulin urease (IgY-urease) suppressed *H.pylori* infection in human after consumption of drinking yogurt fertified with IgY - urease. In addition, it was found that urease breath test values significantly decreased in the volunteers tested group. These results indicate that suppression of *H.pylori* infection in humans could be achieved by consumption of drinking fertified with IgY - urease.

4.1.1.12 Distribution of *H.pylori* Infection According to Social Aspects:

Results illustrated in (Table 4-15) show an extremely high prevalence of *H.pylori* infection in the age group of (31 to 40) year (96.5%) and the age group (> 61) year (93.7%).

High prevalence rate of infection in age group over (60) may be attributed to cohort of older persons who had greater exposure to *H.pylori* in the past (Calam, 1997). Whereas high prevalence rate (81%) in the age group ranging from (21 to 30) year. This may be due to the fact that up to 80% of the population in the developing countries is infected in childhood.

A similar result was obtained from a study in Poland by Matysaik and Megraud, (1994) who found that prevalence rate of *H.pylori* infection was (85%) in the ages ranging from (15 to 19) years.

Also high prevalence rate of infection in age group (30-41) year was (96.5%), prevalence of infection varied [illegible] the socioeconomic [illegible] studied popu[illegible] Results showed that in the age group of (11 to 20) year the rate of *H.pylori* infection was (75%) and in the age groups (41 to 50) and (51 to 60) the rate of infection were (71%) and (73.7%), respectively.

A study performed in Slovenia in 1994 showed the prevalence rate of seropositive *H.pylori* infection as (73.5%) in age group (30 to 39) year (Matysial and Megraud, 1994). *H.pylori* infection is common worldwide between and within population groups. However, prevalence vary widely.

Taylor *et al.*, (1995) reported that in some developing countries, most of the population is infected by age (10) years, and infection is universal in mild life. High prevalence of *H.pylori* infection is observed in the elderly, this appears to reflect a birth cohort effect. Older individuals have more infection because they were born at times when infection was more common than it is today.

(Table 4-15) shows that (79) from (89) males (88.7%) and 28 from (41) female (68%) were infected with *H. pylori*. Statistically, there were significant differences in the infection rats at ($p \geq 0.05$) between males and females. Most studies suggested that males and females are infected at approximately the same rate (Mossi *et al*., 1993; Vaira *et al*., 1994; Gasbarrini *et al*., 1995). Zheng *et al*., (2000) found that about (45%) of middle aged British men were infected with *H.pylori* strains.

Frequency of *H.pylori* seropositively increases with age and varies merely from one country to another as from one ethnic population to another within countries. A cohort study carried out by Gill *et al* (1994) in Dunedin South Island showed that only (4%) of (21%) year age group infected by *H. pylori* compared to (79%) for Indian teenager.

high prevalence rates of infection were reported for those with low (85%) and moderate (74%) income.

Malaty and Graham, (1994) showed an inverse relationship existed between *H.pylori* infection and socioeconomic state. Socioeconomic status during childhood may serve as a particularly good indicator of *H.pylori* risk. Poor socioeconomic conditions during childhoods, as measured by household crowding and parental income, are though to play engenders greater risk for infection, with sharing beds during childhood especially hazardous practice (Webb *et al*., 1994).

High prevalence rate of infection with *H.pylori* was found between smoking patients. Results of this study showed that (84%) of the (68) infected patients were smokers. A similar result was obtained by McColl *et al.*, (1997) who found that high prevalence rate of infection with *H.pylori* (67%) was among smoking patients. Smoking was the most powerful acquired factor in predicting infection with *H. pylori*. Smoking seems to act as a major risk factor for ulcer disease rather than a minor. A similar study done by Al-Hadi (2001) which found high prevalence rate (76.9%) of infection among smoking patients.

Fontham *et al.*, (1995) found that smoking factor plays important role in increasing prevalence of infection with *H. pylori*.

High prevalence *H.pylori* infection was observed among alcohol drinking patients with (44) out of (68) infected patients [illegible] *et al.*, (1995) [illegible] *H.pylori* infection among heavy alco[illegible] the non - drinkers which may suggest a vomits / oral route of infection. Reduction of mucosal protective factors may limit *H.pylori* colonization or, less likely, that alcohol-induced gastritis is a prerequisite of infection.

Statistical results indicate significant differences between *H.pylori* infection and consuming alcohol at p-value ($\geq$ 0.05). Hauge *et al.*, (1994) found that there is no consensus on the role of alcohol in the development of *H.pylori* related to peptic ulcer disease. They stated that alcohol consumption also damages the mucosal protection.

Table (4-15): Distribution of Infection According to Sex, Age, Socioeconomic State and Different Individual Behaviors.

	No. of Infection (%)	Total No.	X^2	P-value
Sex				
Male	79 (88.71)	89	8.078	P = 0.004
Female	28 (68)	41		
Age				
11-20	21 (81)	26		
21-30	28 (96.5)	29		
31-40	17 (71)	24	16.656	0.000
41-50	[illegible]	[illegible]		
51-60	15 (93.7)	16		
> 61	12 (75)	16		
Socioeconomic State				
Low	85 (85)	100		
Moderate	14 (74)	19	16.656	0.000
High	8 (73)	11		
Smoking				
Smoker	68 (84)	81	0.664	0.415
Non smoker	39 (79.5)	49	6.034	0.014
Drinking Alcohol				
Drinker	44 (75.6)	58	15.586	0.000
Non drinker	15 (20.8)	72		

4.1.1.14 Relation Between *H.pylori* and Associated Diseases:

Helicobacter pylori infection is statically associated with several conditions outside the digestive tract. Among them are coronary heart disease, iron deficiency, anemia and cot death (Bateson, 2000).

(Table 4-16), showed an association is existed between several diseases and *H.pylori* infection. Of the 7 patients with colilts, 5 (71%) were found to be infected with *H.pylori* followed by (4) out of (7) gallstone patients (57%), (2) out of (5) rheumatoid arthritis (40%) and (4) out of (10) anemia patients (40%). While patients suffering from growth retardation didn't showed any infection by *H.pylori.* Moreover, *H.pylori* infection has been found among myocardic infraction, hypertension and diabetic mellitus patients at (36%), (27%) and (50%) of the investigated patients gave positive results, respectively.

Table (4-16): Relation between *H.pylori* and Associated Digestive Disease among Dyspeptic Patients.

Disease	Total No.	Infected No.	Infected (%)
Colitis	7	5	71
Gallstone	7	4	57
Rheumatoid arthritis	5	2	40
Anemia	10	4	40
Myocardic infarction	11	4	36
Hypertension	22	6	27
Diabetic mellitus	12	6	50
Growth retardation	3	-	-

Wingcup *et al.,* (1996) reported a high rate of *H.pylori* infection among myocardial infraction patients and in (137) cases of strokes. Another study done by Patel *et al.,* (1995) found that out of (47) men suffering ischemia or infraction, 36 (76%) had antibodies to *H. pylori*.

A recent study done by Al-Rawy (2005), found that (60%) of peptic ulcer patients with diabetic were found to be infected by *H. pylori*. Candelli *et al*., (2003) demonstrated higher seroprevalence of *H.pylori* infection among diabetics with increased production of cytokines that alter the control of glycemia in diabetic patients due to stimulate secretion of insulin - counter regulatory hormones that affect carbohydrate metabolism (McMahon and Distrain, 1995).

Other studies demonstrated associations between *H.pylori* and each of hypertension and myocardial infraction (Tebbe *et al.,* 1996). Harkone[illegible] et al. (2001) found that [illegible] in vitamin B12 is commonly referre[illegible] gastritis caused by *H.pylori* infection in antrum and corpus area of the stomach.

4.1.2 Animal Groups:-

Understanding the route of *Helicobacter pylori* transmission is important for public health measures to prevent its spread. One of the suggested theories is transmission from animals to human beings was proposed through an interesting paper from Sardinia and United State performed by Dore (1999) who found that milk and gastric tissue from sheep were cultured and analyzed by PCR. *H.pylori* was demonstrated in (60%) (38/63) of milk samples and in (30%) (6/30) of sheep tissue samples.

In the present study, a total of (170) animals (20) bovine and (100) sheep gastric biopsy tissues as well as (50) raw sheep milk) were investigated for presence of *H.pylori* using different culture media, biopsy urease test and histological examination methods. *H.pylori* was not isolated in any of the animal samples as shown in the sectioning of sheep gastric tissue (Figure 4-21).

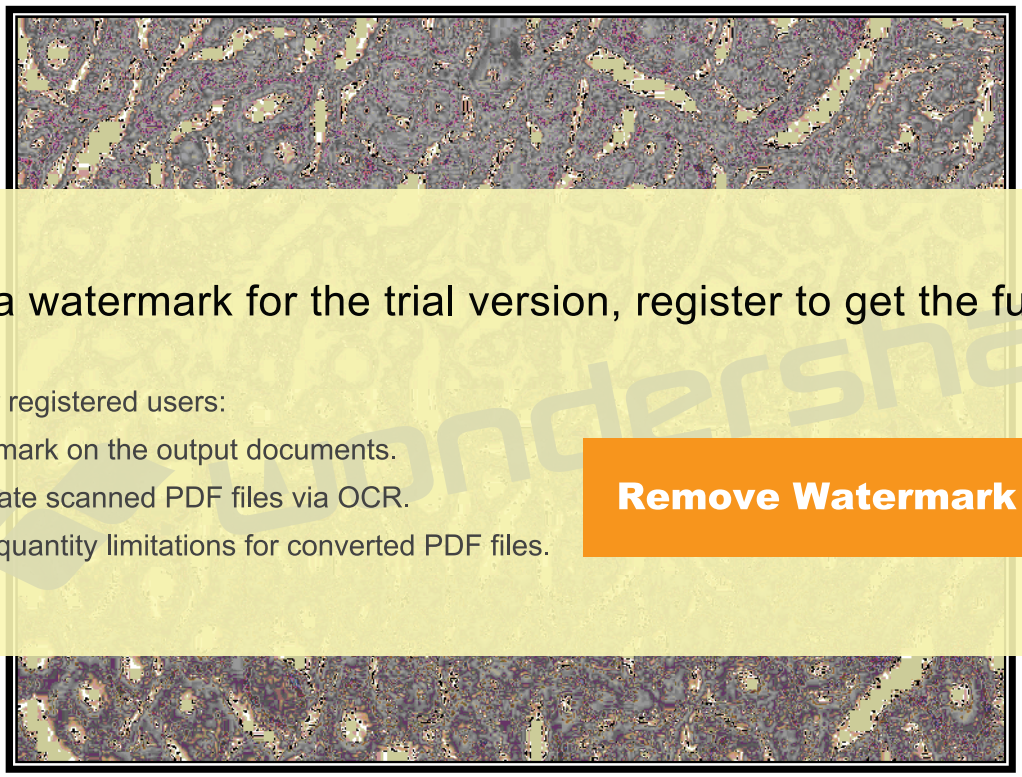


Figure (4-21): Histological Sectioning Showed Normal Sheep Gastric Tissue.

Results agreed with those obtained by Turutogln and Mudul, (2002) who found that *H.pylori* was not isolated from any of the (440) raw sheep milk samples commonly consumed as human food in Burdur region of Turkey.

Furthermore, a similar results were obtained by Bohimler *et al.*, (1996) who examined (177) samples of udder secretion from cows with mastitis, (199) samples of milk from healthy cows and (100) chicken stomachs, and found that non of these samples contained *H.pylori*.

In contrast, Fujimura *et al.*, (2002) demonstrated the *ure* A gene of *H.pylori* in 13 of 18 (72.2%) raw cow milk samples, and in (11) of (20) (55%) commercial pasteurized milk samples. Furthermore, *H.pylori* was cultured in one raw milk sample instead of a pasteurized milk sample. A study reported *H.pylori* in commercial vendor cats led to a suggestion that *H.pylori* may be a zoontic pathogen with transmission occurring from cats to humans (Handt *et al.*, 1994). Van Duynhoven *et al.*, (2001) reported

macaque monkeys. This may be related to the contact between humans and these animals which usually more [illegible] important role in the transmission to [illegible] inability to isolate the organism from other animals may be due to the difficulty of detecting the bacterium in materials other than gastric tissue (Fox, 1995).

Chapter Five

Conclusion

1- *Helicobacter pylori* infection was highly prevalent among dyspeptic patients.

2- Combination of multiple diagnosis methods allows the diagnosis of *H.pylori* immediately after endoscopy and allows an earlier appropriate treatment.

3- Histological section of gastric biopsy specimens stained with Hematoxyline and Eosin and Giemsa stains were found to be the most sensitive test to detect *H.pylori* infection followed by urease

4- Culturing method has the highest specificity and lowest sensitivity

5- Brain - heart infusion agar was the superior medium for isolation of *H.pylori* compared to Columbia and Brucella agar.

6- Inability to isolate the *H.pylori* from some domestic animals indicated that *H.pylori* is the major human gastrointestinal pathogen, and human stomach is the only known reservoir for this organism.

7- High prevalence of antibiotic resistance was observed among *H.pylori* isolates especially to amoxicillin and metronidazole.

8- Significant differences in the infection rates were recorded between males and females, also between alcohol consumers.

9- *Lactobacillus acidophilus* has considerable inhibitory effects against the tested *H.pylori* isolates.

Recommendation:

1- Studying the role of probiotics in association with antibiotics for the treatment of *H.pylori.*

2- Further randomized and controlled studies to investigate the route of transmission of *H.pylori.*

3- Performing of molecular study to understand the virulence factors of *H.pylori* such as the flagella, urease, adhesion and cytotoxin protein.

4- Extened studies are needed to identify the carcinogenic effect of *H.pylori* infection such as reduction of gastric antioxidant absorbed by *H.pylori* infection or stimulation of epithelial proliferation.

Acknowledgment

At the beginning, thanks to great ALLAH who gave me the reality and strength to accomplish this work.

I would like to express me sincere thanks and appreciation to my supervisors ***Professor Dr. Abdul W.Baqir and Professor Dr.Makki H.Fayat*** *for their continuous support and valuable advice during the whole period of my study. A word of deep thanks, appreciated and gratitude to the Dean of Science College and chairman of Biotechnology department for their consent and support my post graduate study.*

My special thanks are due to my colleague Zeenah who was always therefor me.

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Maysaa

Kit Contents, Reagent Preperation and Material Provided:-

- **Microplate:** 12 × 8 strips in frame coated with partially purified *H.pylori* bacterial antigen.
- **Washing Buffer:** Phosphate buffer contianing Tween 20 and preservative.
- **Diluent Buffer:** Phosphate buffer contianing protien, Tween 20, preservative and red dye extract.
- **Calibrator:** One vial containing 1.5 ml of human serum- based *H.pylori* IgG calibrator with preservative.
- **Negative Control:** One vial containing 1.5 ml of human serum-based *H.pylori* IgG negative control with preservative.
- **Positive Control:** One vial containing 1.5 ml of human serum-based *H.pylori* IgG positive control with preservative.
- **Conjugate Solution:** 0.5 ml of monoclonal anti-human IgG in stabi
- **Substrate Solution:** 15 ml oftetramethylbenzidine in buffer containing preservative.
- **Stop Solution:** 15 ml of 0.1mol/l sulphuric acid.
- **Incubation Covers:** Four plastic sheets to cover the microplate during incubation.

بسم الله الرحمن الرحيم

وَلَوْلَا فَضْلُ اللَّهِ عَلَيْكَ وَرَحْمَتُهُ لَهَمَّت طَّآئِفَةٌ مِّنْهُمْ

صدق الله العظيم

سورة النساء

الآية {١١٣}

Supervisor Certification

I certify that this dissertation was prepared under my supervision in the College of Science, Al - Nahrain University as partial requirements for the degree of Doctor of Philosophy in Biotechnology.

Signature
Supervisor: Dr. Abdul W. Baqir
Scientific Degree: Professor
Date:

Signature
Supervisor: Dr. Makki H. Fayat
Scientific Degree: Professor
Date:

Signature
Supervisor: Dr. Nabeel K. Al-Ani
Scientific Degree: Assistant Professor
Title: Head of Biotechnology Department
Date:

Committee Certification

We, the examining committee, certify that we have read this dissertation and examined the student in its contents and that, according to our opinion, is accepted as a dissertation for the degree of Doctor of Philosophy in Biotechnology.

Signature
Name: Dr.
Scientific Degree:
Date:

(Chairman)

Name: Dr.
Scientific Degree:
Date:

(Member)

Name: Dr.
Scientific Degree:
Date:

(Member)

I, hereby certify upon the decision of the examining committee.

Signature
Supervisor: Dr. Laith A. Z. Al-Ani
Scientific Degree: Assistant Professor
Title: Dean of College of Science
Date:

Table of Contents

List of Tables

List of Figures

List of Abbreviations

Cag: Cytotoxin - Associated Gene.

µg/ml: Microgram per milliliter.

BHI-VAN: Brain Heart Infusion Agar-Vancomycin, Amphotricin and alidixic Acid.

CLO: Campylobacter Like Organism.

Conc.: Concetration.

CSG: Chronic Superficial Gastritis.

EGD: Esophogastroduodenal Endoscopy

EIU: Enzyme Immuno Unit.

ELISA: Enzyme Linked Immunosorbant Assay.

GI: Gastrointestinal

H&E: Hematoxyline and Eosin.

H.pylori: *Helicobacter pylori.*

HsP A,B: Heat Shock Protein A and B.

KD: Kilo Dalton.

M.B: Methyline Blue.

MALT: Mucosa Associated Lymphoid Tissue.

MALT: Mucosa Associated Lymphoid Tissue Lymphoma

Mg/L: Microgram per Liter.

MIC: Minimun Inhibitory Concetration.

MRS: DeMan Regosa Sharpe.

NSAIDs: Non - Steroidal Anti Inflammatory Drugs.

NUD: Non Ulcer Dyspepsia.

OiPA: Outer Membrane Inlammatoy Protien.

PBS: Phosphate Buffer Saline.

PCR: Polymerase Chain Reaction.

UEP: Uroepithelial Cell.

Vac: Vacuolating Cytotoxin A.

Dedication

To the great Allah for all his Giftness

To you my lovely… … mother

To you my dearest… …father

I dedicate this work

Maysaa

Republic of Iraq
Ministry of Higher Education
and Scientific Research
Al-Nahrain University
College of Science

Evaluation of Different Methods for Detecting *Helicobacter pylori* Isolated from Human and the Effect of Probiotics on the Bacterial Growth

Dissertation

Submitted to the College of Science
In Partial Fulfillment of the Requirements for the Degree
of Doctor of Philosophy in Biotechnology

By

Maysaa Ghasib Al-yas

B.Sc. Biotechnology / Al-Nahrain Univ./ 2000

M.Sc. Biotechnology / Al-Nahrain Univ./ 2002

April
2006

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Summary:

This study included two different grouping samples (human and some domestic animals). In human group, (130) dyspeptic patients were subjected to esophageal gastroduodenoscopy and gastric biopsy specimens were taken from the antrum and body were applied for microbiological analysis which included: urease test, bacterial culture using different culture media, direct biopsy smear examination using Giemsa and Gram stain as well as histological test for detecting *Helicobacter pylori* using Hematoxyline, Eosin (H&E) and Giemsa stains. Venous blood samples were also collected randomly from (75) patients for enzyme linked immunosorbant assay (ELISA test) and (15) healthy persons with no history of dyspepsia or peptic ulcer disease were used as control.

Results showed that occurrence of *H.pylori* was (81.5%) among [illegible] was a signific[illegible] and duodenitis (89%), gastric erosion (83%), duodenal ulcer (81%) and gastritis disease (75.6 %).

Results also showed that using modified selective medium was better than the nonselective for primary isolation of bacteria, with a recovery rate of (24%) by using brain-heart infusion agar and (6.2%) for the nonselective blood agar.

Histological investigation using Giemsa and (H&E) stains were proved to be the most sensitive method for detecting *H.pylori* infection with a sensitivity of (98%), while the sensitivity of bacterial identification by the biopsy urease test, direct biopsy smear examination and culture of biopsy specimens were (95%), (80%) and (29%) respectively.

Statistical analysis of the ELISA results showed that there were significance differences in the mean of immunoglobulin G (IgG) specific antibodies concentration observed among males and females of the gastric cancer group compared with the controls, while no significant differences were revealed among males and females of other groups.

When the isolates of *H.pylori* were subjected to the sensitivity test against (12) antibiotics, results showed that ciprofloxacin was the most effective antibiotic against the isolates when the sensitivity percentage was (62%). Penicillin G, ampicillin, cloxicillin and amoxicillin on the other hand, were the least effective antibiotics when bacterial sensitivity ranged between (8%) and (37%).

Depending on the antibiotic sensitivity results, five isolates of *H.pylori* were selected (due to their multiple resistances to antibiotics) for determining the minimal inhibitory concentration (MIC) of some [illegible] bacteria [illegible] concentration (512µg/ml) of penicillin G, concentration (265µg/ml) of metronidazole, with slight resistance for clarithromcin and tetracycline (32µg/ml).

When inhibitory activity of lactic acid bacteria (LAB) isolates against *H.pylori* was tested on solid medium, less inhibitory activity against it, was detected compared to the liquid medium, the inhibitory activity increased after incubation periods of (48) and (72) hours.

Minimum inhibitory concentration (MIC) of LAB concentrated filtrate was estimated. Results showed that (50%) and (60%) concentrations of such filtrates were effective in the inhibition of *H.pylori* growth. MICs were determined for the four - fold concentrated filtrates of *Lactobacillus acidophilus* against adhesion property of *H.pylori* HP1, HP2 and HP3. Results showed that filtrates were able

to lower adhesion of pathogenic bacteria *H.pylori* to the epithelial cells when the average of adherence decreased to (5-15 bacteria/ cells) instead of (60-75 bacterial/ cell) recorded by HP1, (70-85 bacterial/ cell) by HP2 and (80-95 bacterial/ cell) obtained by HP3 before treatment. Also an interesting finding upon probiotic treatment against *H.pylori* isolates with decreased in urease activity was observed when a color change was detected after (10, 18 and 24) hr; rather than few minutes before treatment. This could be due to lactic acid and other inhibitory substances produced in concentrated filtrate by LAB isolates. A transformational change was detected when a coccoid form of *H.pylori* was observed after treatment with LAB isolate.

In the animal group, a total of (170) samples of animal (20 bovine and 100 sheep gastric biopsies as well as 50 raw sheep milk samples) were collected from Sheikh Maroof and Al-Doora slaughtering houses in Baghdad [illegible] the presence [illegible]. Different [illegible] media, procedures and histological examin[illegible]

Results showed that *H.pylori* was not present in any of these samples.

Table (4-11): Antibiotic Susceptibility of *H. pylori* Rapid Urease Producer Isolates Determined by Diameter of Inhibition Zone (mm).

Isolate	CRO 30 meg	AMX 20 meg	AK 30 meg	CRO 30 meg	AMP 10 meg	CX 1 meg	Crr 15 meg	Mt 5 meg	PG 10 meg	TE 30 meg	E 30 meg	CIP 30 meg
HP1	R	R	R	R	R	R	R	R	R	R	S	S
HP2	R	R	R	R	R	R	R	R	R	R	R	S
HP3	R	R	R	R	R	R	R	R	R	R	R	R
HP4	S	S	R	R	R	S	R	R	R	R	R	S
HP5	R	R	R	R	R	R	R	R	R	R	R	R
HP6	R	R	R	R	R	R	R	R	R	R	R	R
HP7	R	R	R	R	R	R	R	R	R	R	S	R
HP8	S	S	S	R	R	R	S	R	R	S	S	S
HP9	R	R	R	S	R	R	R	S	R	R	R	R
HP10	R	R	R	R	R	R	S	R	R	R	R	S
HP11	S	S	R	S	S	R	S	S	R	R	S	S
HP12	R	S	R	S	R	S	R	S	S	R	S	S
HP13	R	R	S	R	R	R	S	R	R	R	S	S

الخُلاصـة:-

شملت الدراسة هذه على مجموعتين مختلفتين من النماذج (من الانسان ومن بعض الحيوانات الأليفة). شملت مجموعة الانسان (130 مريضاً) يعانون من عسر الهضم اذ اخضع جميعهم الى التنظير المعدي العفجي وتم أستئصال الخزعات النسيجية من غار وجسم المعدة لأجراء الاختبارات المايكروبايولوجية عليها والتي شملت اختبار انتاج انزيم اليوريز، الزرع البكتيري بأستخدام أوساط زرعية مختلفة، اختبار المسحة المباشرة والاختبار النسيجي للكشف عن بكتريا *Helicobacter pylori* بأستخدام صبغتي الهيماتوكسلين والايوسين و كمزا. كما تم سحب عينات دم وريدي من (75) مريض عشوائيا لأجراء بعض الفحوصات المناعية (الاليزا)، بينما أستخدم (15) شخصاً صحيحاً لم يخضعوا للتنظير المعدي العفجي ممن لم يكونوا قد عانوا من عسر هضم (كمجموعة سيطرة).

أظهرت النتائج ان نسبة تواجد بكتريا *H.pylori* في المرضى (81.5%) من الذين يعانون من عسر الهضم، وكانت هناك علاقة بين الخمج ببكتريا *H. pylori* والتهاب الاثنى عشر بنسبة (89%) ومع تقرح المعدة (83%) ومع قرحة الاثنى عشر (81%) ومع التهاب المعدة (75%). [illegible] هناك فروق معنوية بين العوامل الاجتماعية التي ساعدت على اكتساب الاصابة اذ كان [illegible] الجنس [illegible] ($p < 0.05$) والحالة الاقتصادية [illegible] انتقائي للعزل الاولي للبكتريا اذ بلغت نسبة الكشف بأستخدام أغار نقيع الدماغ القلب الانتقائي (24%) و(6.2%) عند أستخدام أغار الدم غير الانتقائي.

وجد لدى التحري عن بكتريا *H.pylori* في المقاطع النسيجية بإستخدام صبغتي الهيماتوكسلين – الايوسين و كمزا انهما أفضل طريقة للكشف عن الاصابة عندما بلغت حساسيته (98%)، فيما بلغت حساسية اختبار التشخيص البكتيري للخزعة النسيجية بأستخدام اختبار إنتاج اليوريز، إختبار المسحة المباشرة والزرع البكتيري (95%)، (80%) و(29%) على التوالي.

عند اخضاع بكتريا *H.pylori* لإختبار الحساسية اتجاه (12) مضاداً حياتياً، أظهرت النتائج ان السبروفلوكساين كان اكثر المضادات تأثيراً عندما لم تتجاوز نسبة المقاومة له (38%)، بينما كان البنسلين، الامبسلين، كلوكسلين والاموكسلين أقل تأثيراً منه إذ تراوحت نسبة مقاومتها مابين (92%) و(62%) على التوالي.

إعتماداً على نتائج فحص الحساسية للمضادات الحياتية فقد أختيرت خمسة عزلات من بكتريا *H.pylori* من التي اظهرت صفة المقاومة المتعددة للمضادات الحياتية. ودلت نتائج

تقـدير التراكيـز المثبطـة الـدنيا لـبعض المركبـات الحياتيـة ان للعـزلات هـذه قابليـة علـى النمـو في تراكيز عالية (٥١٢ ملغم / مل) لمضادي البنسلين والامبسلين وتراكيز معتدلة للمترونـدازول (٢٥٦ ملغم /مل) بينما أظهرت العزلات مقاومة قليلة للكلاثرومايسين وتتراساكلين (32 ملغم / مل).

لـــدى اختبـــار الفعاليـــة التثبيطيـــة لاحـــدى عـــزلات بكتيريـــا حـــامض اللاكتيـــك (*Lactobacillus acidophilus*) ضد ثلاث عزلات من بكتريا *H.pylori* على كل من الوسط الصلب والسائل، وسجلت اقل فعالية تثبيطيه على الوسط الصلب مقارنة بالوسط السائل الذي أعطى أفضل فعالية تثبيطية ولاسيما عند زيادة فترة الحضانة الى (48 و72) ساعة. كما وتم تقدير التراكيز المثبط الادنى للراشح المركز ووجد ان تركيز الرواشح بنسب (50%) و(60%) كانا الافضل في اعطاء فعالية تثبيطية لتثبيط نمو بكتريا *H.pylori*.

الشـكلي لبكتريـا *H.pylori* بعـد معاملتهـا براشـح بكتريـا حـامض اللاكتيـك مـن خـلال ملاحظـة الأشكال المكورة والدائرية أثناء فحصها بالمجهر الضوئي.

أما بالنسبة لمجموعة الحيوانات الأليفة فقد شملت أخذ (170) عينة حيوانية (20 خزعة نسيجية من أمعاء البقر، (100) خزعة نسيجية من امعاء الغنم و(50) عينة حليب غنم غير مبستر)، جمعت النماذج من مجزرتي شيخ معروف والدورة في بغداد واخضعت الخزعات النسيجية الى إختبار إنتاج اليوريز، الزرع البكتيري باستخدام أوساط مختلفة والاختبار النسيجي، أظهرت النتائج عدم وجود بكتريا *H.pylori* في أي من هذه العينات.

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تقييم طرق مختلفة للكشف عن *Helicobacter pylori* معزولة من الانسان، وتأثير الكائنات المنافسة (بروباوتك) على النمو البكتيري.

رسالة مقدمة الى
كلية العلوم جامعة النهرين
كجزء من متطلبات نيل درجة الدكتوراه فلسفة
في التقانة الاحيائية

من قبل

ميساء جاسب الياس

بكالوريوس تقانة احيائية/ جامعة النهرين/ ٢٠٠٠

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ربيع الاول
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نيسان
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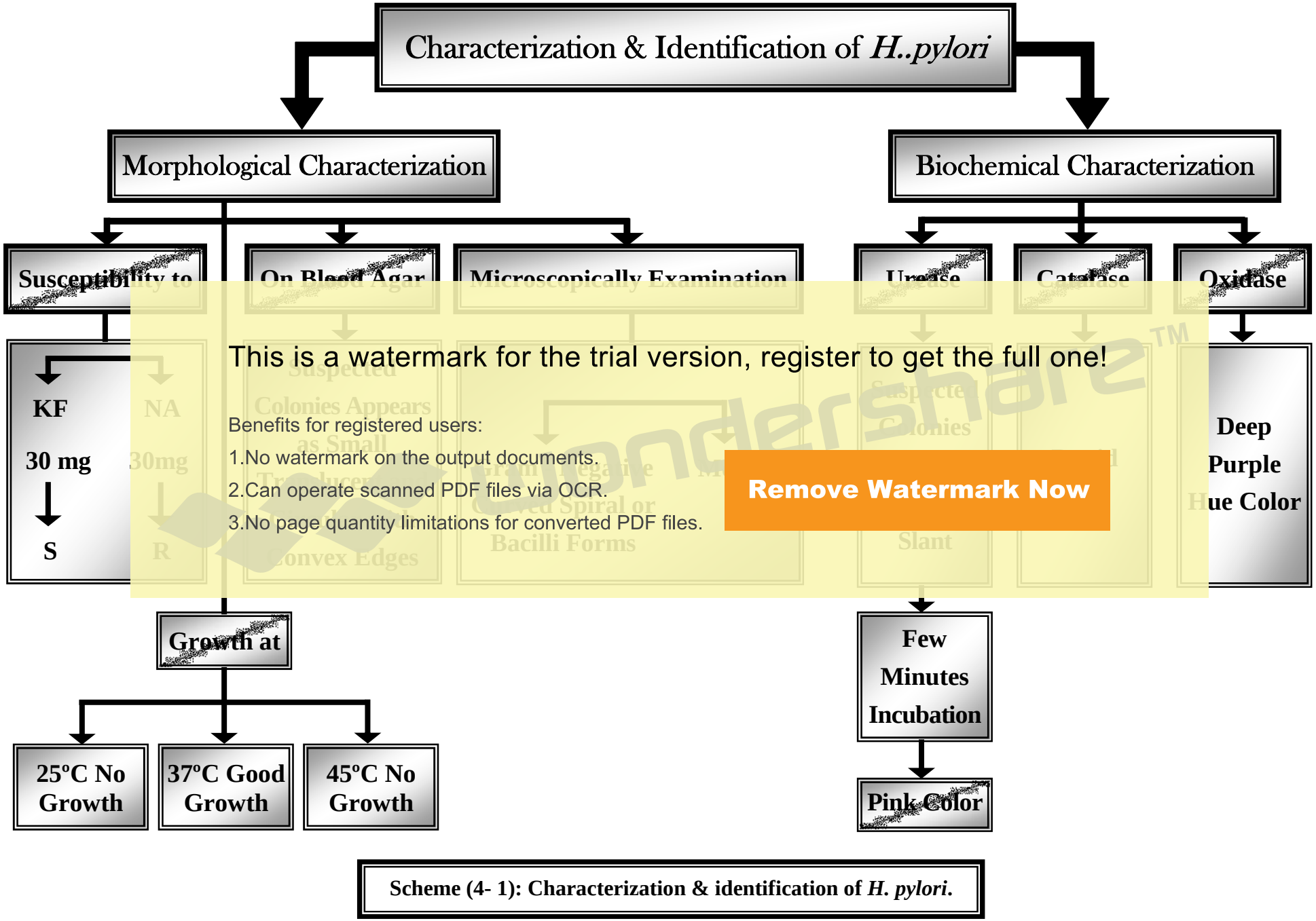

Scheme (4- 1): Characterization & identification of *H. pylori*.

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Chapter One

Chapter Two

Chapter Three

Chapter Four

Results and Discussion

Chapter Five

Conclusion and Recommendation