DETECTION OF HELICOBACTER PYLORI IN GASTRIC BIOPSY SAMPLE FROM PATIENTS WITH UPPER GASTROINTESTINAL DISORDERS USING CONVENTIONAL AND MOLECULAR METHODS

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INTRODUCTION

The discovery of *Helicobacter pylori* in 1982 by Marshall & Warren\(^{118}\) was the starting point of a revolution concerning the concepts and management of gastroduodenal diseases. *H.pylori* is a gram negative curved motile rod found in the deeper portion of the mucous gel coating the gastric mucosa. It is extraordinary among bacteria in its ability to colonize and persist among this niche for decades despite host defenses and gastric acidity.

It is now established that *H.pylori* infection induces several gastrointestinal complications, ranging from mild gastritis to peptic ulcers\(^{85}\) and even gastric malignancies, such that the International Agency for Research on Cancer\(^{38}\) has declared this pathogen as an independent carcinogen. In addition the etiologic association of this infection with an increasing number of disorders including cardiovascular diseases\(^{27}\), and metabolic syndrome\(^{113}\) are being investigated. Therefore it is of utmost essence to detect the infection and pursue with eradication therapy and follow up.

Several diagnostic modalities are available to detect *H.pylori* infection. The two major categories of diagnostic assays are endoscopic or invasive tests and non endoscopic or non invasive tests. The endoscopy based tests include rapid urease, histopathological evaluation, polymerase chain reaction and culture. The non-endoscopic tests include antibody detection methods and carbon labeled urea breath test. The choice of the tests depends on the laboratory resources and the clinical situation whether for diagnosing infection or for documenting eradication.
*H. pylori* is a robust producer of urease and its presence is detected by rapid urease tests. The advantage of these tests is that they can be readily performed in the endoscope suite. Another rapid test is smear evaluation of the specimen. Imprint smears stained by rapid Giemsa or Gram stain provide an adjuvant to histopathological examination of gastric biopsy specimens.

Culture is probably the most difficult approach to the diagnosis of *H. pylori*. The advantages are that it is highly specific and the antibiotic sensitivity can be detected. High rate of false negatives due to the fastidious nature of the organism coupled with expense incurred for culture have limited its application.

Chronic *H. pylori* infection elicits local and systemic immune response that lead to production of antibodies. The presence of IgG antibodies to *H. pylori* can be detected by immunoassays. Serology is sensitive for primary diagnosis but is not useful in assessing post treatment *H. pylori* status58.

The urea breath test relies on the urease activity of *H. pylori* to detect the presence of infection. Sensitivity is excellent because the whole stomach is sampled. Unlike serology it is useful for determining the success of the eradication therapy. Even though the test is more accurate than serology its usage is limited due to high cost and lack of facilities for testing.

With the advent of PCR, many possibilities have emerged for diagnosing *H. pylori* infection. PCR allows identification of the organism in samples with few bacteria and it has been successfully used to detect *H. pylori* CagA and VacA virulence genes in gastric biopsy samples100. PCR is being
evaluated for its utility in identifying *H. pylori* in samples of dental plaque, saliva and other easily sampled tissues. The potential advantage of PCR includes high specificity, quick results and the ability to identify different strains of bacteria for pathogenic and epidemiologic studies. The major limitation of PCR is that relatively few laboratories currently have the capacity to run the assay.

In the present study, four parameters, namely rapid urease test, culture method, histopathology, and serology were used to detect presence of *H. pylori* in the gastric biopsy samples, supplemented with PCR for selected 50 samples.
REVIEW OF LITERATURE

Historical perspective

The presence of gastric spiral bacteria was first reported in 1893. Spiral bacteria were demonstrated for the first time, in the human stomach in 1906. In 1924 the presence of urease activity in the human stomach was documented. The bacterial source of gastric urease was confirmed in 1968. Bacteria were reported in association with gastritis in 80% of gastric resection specimens from patients with gastric ulceration.

The modern era was heralded in 1981 when Barry Marshall, began a clinical research project with Robin Warren, in the Royal Perth Hospital, Western Australia. Subsequent attempts to culture these bacilli were unsuccessful until April 1982 when during the Easter weekend, the plates were unintentionally incubated for 5 days and colonies were visible.

The association of these bacteria with gastritis was first presented at the Royal Australian College of Physicians on 22 October 1982 and published in letter form in 1983.

These 'Campylobacter'-like organisms' were called Campylobacter pyloridis. For grammatical reasons, the name was changed to Campylobacter pylori in 1987. Subsequently, it was shown that C pylori did not belong to the genus Campylobacter and a new genus name was suggested in 1989.65
The association with peptic ulceration, and possibly with gastric adenocarcinoma, was initially suggested by Marshall et al.\textsuperscript{67}.

Reliable diagnostic techniques which facilitated epidemiological and interventional studies, such as serology, the rapid urease test, and the [13C] and [14C] urea breath tests soon became available.

A group of specialists with an interest in the infection, the European \textit{H. pylori} Study Group, was formed in Copenhagen in 1987 and this was followed by an exponential increase into the study of the role of the bacteria in gastro-duodenal diseases. The first long-term clinical trial of treatment aimed at eradicating \textit{H. pylori} in patients with duodenal ulceration was reported in 1987.

The relationship between the organism and gastric adenocarcinoma and MALT (gastric mucosa-associated lymphoid tissue) lymphoma was reported in 1991.

Subsequent studies assessed the role of the organism in gastro-oesophageal reflux disease, patients receiving long-term acid suppressing medication, paediatric populations, and non-ulcer dyspepsia.

In 1994, \textit{H. pylori} was recognized as a grade I (definite) carcinogen and the National Institutes of Health Consensus Development Conference Statement recommended that all patients who are found to have gastric or duodenal ulceration and concurrent \textit{H. pylori} infection should receive treatment aimed at eradicating the bacterium\textsuperscript{81}. 
In 1997, it was 'strongly recommended' by a European consensus panel that patients with *H.pylori* infection and peptic ulcer, low grade mucosa-associated lymphoid tissue lymphoma, severe macroscopic or microscopic gastritis or recently resected early gastric cancer should receive a proton pump inhibitor-based triple therapy to eradicate the infection.

**Definition of genus**

Helicobacters are helical, S-shaped or curved gram-negative rods, 0.5-1.0 µm wide by 2.5-5.0µm long. Motility is rapid and darting by means of single or multiple unipolar, bipolar, or lateral sheathed flagella. Cells exposed to air may form coccoid bodies. They are non-sporing and micro-aerophilic with respiratory metabolism. They are non-saccharolytic, oxidase-positive and catalase-positive, except for Helicobacter canis which is catalase-negative\(^{29}\).

**Habitat**

The surface of the human stomach mucosa is the major habitat of *H. pylori*. Its natural niche appears to be the mucous- lined surface of the non-acid-secreting area close to the pyloric sphincter, i.e., the antrum\(^3\).

Almost all isolations are from gastric biopsy specimens, but the organism has occasionally been detected in gastric juices, saliva, dental plaque\(^{50}\), bile and faeces.
Morphology

*H. pylori* cells take the form of curved, or S-shaped gram-negative rods, 0.5-0.9 μm wide by about 3 μm long with a wavelength of about 2.6 μm. In agar cultures spiral forms are less obvious and cells appear more as singly curved rods. *H pylori* undergo coccoid transformation on exposure to air within 1-2 hr at room temperature, and in this state it fails to grow on subculture. Such coccoid forms do not appear to be virulent (Eaton et al. 1995).

By electron microscopy, the organism is spiral with bluntly rounded ends and with 4-8 sheathed, unipolar, flagella; the sheath is continuous with the outer membrane of the cell wall. Some flagella have a terminal bulb. A glycocalyx-like material surrounding the cell is also apparent.

Cultural Characteristics and Growth Requirements

*H. pylori* are strictly micro-aerophilic, requiring CO₂ (5-20%) and high humidity for growth. *H. pylori* require media containing supplements such as blood, haemin, serum, starch or charcoal. Growth is best on media such as moist freshly prepared heated (chocolated) blood agar, or brain-heart infusion agar with 5% horse blood and 1% IsoVitaleX.

Strains grow in various liquid media supplemented with fetal calf or horse serum (Shahamat et al. 1991). Some strains grow in serum-free media, notably bisulphite-free brucella broth (Hawrylik et al. 1994). All strains grow at 37°C, some grow poorly at 30°C and 42°C [18] but none grows at 25°C. Colonies from primary cultures at 37°C usually take 3-5 days to appear and are
circular, convex and they seldom grow bigger than 2 mm in diameter even if incubation is extended beyond 1 week.

They are weakly hemolytic on 5% horse blood agar. Motility is weak or absent when grown on agar. *H. Pylori* are inactive in most conventional biochemical tests. Notable exceptions are the strong production of urease$^{71}$, catalase$^{67}$ and alkaline phosphatase. All strains produce DNAase, leucine aminopeptidase, and glutamylaminopeptidase (McNulty and Dent 1989)$^{72}$.

**GENETICS**

*H.pylori* has a small genome of about 1700 kb. It is so genetically diverse that, usually, any 2 independent isolates can be distinguished by a variety of genetic analyses, and ribotyping *H.pylori* reveals the presence of two copies of 16S rRNA gene which helped in the classification of the organism.

The presence of virulence genes in *H.pylori* plays a major role in the clinical outcome. *CagA* pathogenicity island is associated with increased incidence of peptic ulcer$^{19}$ and adenocarcinoma$^{12}$. The *VacA* gene is seen in all *H.pylori* strains has been associated with increased gastric damage$^{10}$.

*VacAs1* and *vacAs2* code for toxigenic and non toxigenic types respectively Other important genes are babA1,babA2 and babB which code for adhesion molecule,flagellin genes flaA and flaB,iceA1 a gene for restriction endonuclease, OipA that codes for a protein which induces IL-8 and, genes ureA,ureB,E,F,G that code for urease$^{21}$.
Cell Wall Composition And Antigenic Structure

Helicobacters have the typical cell wall structure of gram-negative bacteria. The fatty acid profile of *H. pylori* is distinctive and is characterized by long-chain fatty acids composed predominantly of tetra-decanoic (14:0) and 19-carbon cyclopropane (19:0 cyc) acids (Moran 1995).

*H. Pylori* contain at least 4 outer-membrane proteins ranging from 48 to 67 kDa that have pore-forming ability. The various strains share the same lipopolysaccaride core antigens but differ in their side chain antigens.

Susceptibility to antimicrobial agents

*H. Pylori* is sensitive to penicillins,(including benzylpenicillin) cephalosporins, tetracycline, erythromycin, rifampicin, amino glycosides and nitro furans, but resistant to nalidixic acid, though sensitive to the more active quinolones such as ciprofloxacin. *H. pylori* is usually susceptible to metronidazole, and are sensitive to colloidal bismuth compounds commonly prescribed for gastric disease in concentrations easily attainable in the stomach. The proton pump inhibitor omeprazole has mild in vitro activity against *H pylori*; 1% bile salts are also inhibitory.

Epidemiology of Helicobacter

Infectious dose

Data concerning infectious dose come from the first successful volunteer infection, in which a dose of $10^5$ organisms in a small liquid feed was used.
It is difficult to judge whether the natural infectious dose of *H. pylori* is large or small. Animal models, especially in primates, and related studies with other Helicobacter\(^{26}\) might help to fill this gap in our knowledge.

**Spread from an animal reservoir or by foods**

*H. pylori* is found in cats\(^ {36}\) and could be a reservoir for human infection\(^ {25}\) but neither a zoonotic reservoir nor food as a vehicle appear to be significantly involved in acquisition of *H. pylori*.

**Spread by water**

Though examples among children in Peru, South America suggest a role for water as a vehicle, it does not project it as a main route of acquisition\(^ {52,5}\).

**Spread by fomites**

Restriction enzyme analysis of bacterial DNA demonstrated individuals infected with identical strains, out breaks of achlorhydria in the U.S and acute mucosal lesion syndrome in Japan are examples of iatrogenic *H. pylori* infection transmitted by endoscope\(^ {114}\).

**Spread by direct contact**

The major modes of transmission of *H. pylori* are still uncertain, although oral-oral, gastro-oral, and faecal-oral routes are all possibilities\(^ {11}\).
There have been suggestions that the mouth may be a reservoir for re-infection, even though samples are often apparently culture negative\textsuperscript{32}. It is also possible that re-infection may occur by person-to-person transmission between spouses\textsuperscript{102}.

A study showed that a physician became infected with \textit{H. pylori} after giving mouth-to-mouth resuscitation to an \textit{H. pylori} positive patient who had recently vomited\textsuperscript{24}. It has been suggested that gastro-oral transmission may be common in children.

Several reports relate to spread from a faecal source. The organism has been cultured from faeces\textsuperscript{111}, and also has been detected in faeces by PCR.

**Variation in the age of acquisition of \textit{H pylori} infection**

The age of acquisition of \textit{H. pylori} shows marked geographical variation. Among children acquisition appears most frequent under 15 years of age\textsuperscript{20}, rates of acquisition in adult life is between 1\% and 3\% per annum\textsuperscript{95}.

**Other risk factors for infection**

Low levels of socio-economic status and education are associated with an increase in prevalence of \textit{H. pylori} infection\textsuperscript{109}. It is possible that consumption of alcohol, may relate to seropositivity. Clustering of infection within families has been commonly, but not uniformly, observed\textsuperscript{22}. High rates of seropositivity in children are found in many developing countries. Seroprevalence of \textit{H. pylori} infection is often similar in males and females\textsuperscript{22}. Several studies found no increased risk of infection in dentists\textsuperscript{62} although
Increased *H. pylori* seropositivity has been noted in gastroenterologists and endoscopists.

**Risk of re-infection**

Many reports define a cut off time, using a minimum period of 4 weeks after treatment, the interval after which they would use the word reinfection. It is probable that many 're-infections' represent recrudescence after treatment failure. Treatment failures were overwhelmingly due to the persistence of the same strain.

**Estimations of a disease to infection ratio for *Helicobacter pylori* – associated peptic ulcer disease**

A disease-to-infection ratio can be estimated for India, where *H. pylori* seroprevalence is high. An endoscopic study found a point prevalence of active peptic ulceration of 4.7% and, if duodenal deformity was included as evidence of previous disease, 11% of the population had had peptic ulcer disease, a lifetime disease risk in infected individuals of 13%. In immigrant monks in South India who neither smoked nor used NSAIDs, the point prevalence of active ulcer disease was 6.6%, the lifetime prevalence (including scarring) was 13.2%, and the lifetime disease-to-infection ratio was estimated at 16%, with a disease-to-infection point prevalence ratio of 8%.

Although the prevalence of peptic ulcer disease varies between populations and over time and began to decline before antibiotic or antacid
treatments became common place, the available data suggest that 6-20% of untreated *H. pylori* infections will lead to peptic ulcer disease.

**Pathogenesis of Clinical Syndromes**

*H pylori* gastritis is associated with several important pathologic conditions including:-

- Duodenal ulcer disease
- Gastric ulcer disease
- Gastric adenocarcinoma arising from the distal stomach.
- Gastric lymphoma

**Duodenal ulcer disease**

Approximately 90% of patients who have duodenal ulceration have *H. pylori* infection. Several characteristics are found more commonly in ulcer-associated than in non-ulcer-associated *H. pylori* strains. The two best characterized are presence of the *Cag* (cytotoxin-associated gene) pathogenicity island, and production of an active vacuolating cytotoxin *Vac A*. The *Cag* pathogenicity island is a genetic region containing over 30 genes. The island encodes a type IV secretory apparatus, best thought of as a syringe, through which one *Cag*-encoded protein, *CagA*, is 'injected' into epithelial cells. *CagA* induces the epithelial cell to undergo several changes including the secretion of pro-inflammatory cytokines, which leads to increased gastric inflammation. *CagA* is also highly immunogenic and anti-*CagA* antibody detection can be used as a serum test for the presence of the island.
The vacuolating cytotoxin, *VacA*, is a pore-forming toxin that increases epithelial permeability and causes massive epithelial cell vacuolation in vitro.

However, only some *VacA* genotypes are associated with the toxigenic phenotype and infection with strains of certain *VacA* genotypes is associated with increased prevalence of peptic ulcer disease.

There has been considerable recent research into other virulence factors include an adhesin, BabA, a bacterial outer membrane proinflammatory protein, OipA, and a restriction enzyme, Ice A, and its associated methylase. Other bacterial factors are also thought to be important for pathogenesis in terms of colonization and the induction of inflammation, such as the enzyme urease and the ability to adhere to gastric mucosa.

**Gastric ulcer disease**

*H. pylori* associated gastric ulcers usually arise in junctional mucosa between antral and corpus type tissues, typically on the lesser curvature. They usually occur in patients with pan-gastritis rather than antral-predominant gastritis and are not associated with increased stimulated acid output. Their pathogenesis is uncertain, but infection with virulent strains and smoking increase risk.

**Gastric adenocarcinoma**

The World Health Organization has classified *H. pylori* as a type 1 or causal carcinogen. It is a risk factor for distal adenocarcinoma with a relative risk of 4-9. Distal gastric adenocarcinoma usually arises in patients with pan-
gastritis. Both \textit{Cag}^+ and cytotoxic strains are more likely to be associated with carcinoma than other strains. Host genetics are also important; people with genetic polymorphisms that lead to high-level secretion of the proinflammatory cytokine interleukin-1 in response to bacterial infection are more likely to develop gastric cancer. Intestinal-type gastric cancer is thought to occur by a step-wise process from superficial gastritis through atrophy to intestinal metaplasia, dysplasia and ultimately carcinoma.

\textbf{Gastric Lymphoma}

Primarily gastric lymphomas arise in lymphoid tissue present in the \textit{H. Pylori} infected stomach. When histologically low grade, the majority regress following \textit{H. pylori} eradication.

The reason for \textit{H.pylori} mediated duodenal ulceration remains unclear. One potential explanation is that gastric metaplasia in the duodenum of Duodenal ulcer patients permits \textit{H. pylori} to bind to it and produce local injury secondary to the host response.

\textit{H. Pylori} antral infection could lead to increased acid production, increased duodenal acid, and mucosal injury. \textit{H Pylori} infection might induce increased acid secretion threw both direct and indirect actions of \textit{H. pylori} and proinflammatory cytokines (IL-8, TNF, and IL-1) on G, D, and pariteal cell. In summary, the final effect of \textit{H. pylori} on the gastrointestinal tract is variable and determined by microbial and host factors.
The type and the distribution of gastritis correlate with the ultimate gastric and duodenal pathology observed. Specifically, the presence of antral-predominant gastric is associated with Duodenal Ulcer formation; gastritis involving primary the corpus predisposes to the development of Gastric Ulcer, gastric atrophy, ultimately Gastric carcinoma.

**CLINICAL FEATURES**

**Acute *H. pylori* infection**

The clinical features of acute infection in the community are unknown. Upper abdominal discomfort and pain occurred 3 days after voluntary dosing\(^ {107} \), followed by vomiting and finally a resolution of symptoms by the end of the week. *Helicobacter pylori* are most commonly acquired in childhood, but whether initial colonization is usually symptomatic or asymptomatic is not known.

**Chronic *H. pylori* infection**

Chronic *Helicobacter pylori* infection is characterized by chronic active gastritis, but this condition is asymptomatic. Chronic infection therefore only, manifest symptomatically if complications develop, such as duodenal ulceration, gastric ulceration or gastric cancer.

**Laboratory Diagnosis**

The two major categories of diagnostic assays for *H. pylori* are endoscopic, or invasive, tests and nonendoscopic, or noninvasive test.
## Diagnostic Test Method of Organism Identification

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### Invasive Tests

The stomach is usually accessed by fiber optic endoscopy, and biopsy specimens are obtained. Using two contrast stains, topical acriflavine and intravenous fluorescein, with a confocal laser endomicroscope, endoscopists were able to detect see clusters *H. pylori* on the surface and in the deeper layer of the gastric epithelium. This technique enabled detection of by surface microscopy imaging of living tissue during ongoing endoscopy for the first time.

It is possible that gastric juice obtained by a nasogastric tube allows the detection of *H. pylori* by culture, staining, urease test, and PCR, but it is less reliable than gastric biopsy specimens. The string test can also be used to obtain gastric mucus, however, the most attractive method seems to be an extendable oro-gastric brush contained in a plastic tube. The brush is
swallowed, extended into the stomach to brush the mucosa three or four times, retracted in the protective sleeve, and withdrawn from the patient. This method is rapid and appears to be reliable for *H. pylori* infection diagnosis \(^{34}\).

**Specimen Collection.**

The best specimens to culture *H. pylori* are biopsy samples obtained during endoscopy. The recommendation is not to consume Proton pump inhibitor for 2 weeks prior to endoscopy.

**Transport of biopsy specimens.**

*H. pylori* are a fragile organism and must be protected from desiccation and contact with oxygen and room temperature. It is mandatory to place them either in a saline solution for short-term transport (4 h maximum)\(^{76}\) or in a transport medium, usually consisting of semisolid agar, maintained at 4°C. A commercially available medium, Porta-germ pylori is effective for this purpose \(^{51}\). Storage at 4°C in a medium containing 20% glycerol also led to *H. pylori* recovery in 81% of the biopsy specimens tested \(^{35}\).

**Grinding of biopsy specimens.**

Comparison of culture performed with and without grinding showed a higher number of colonies after grinding, for this reason grinding of the biopsy specimen is mandatory \(^{32}\).
The media components include an agar base, growth supplements and selective supplements. Most agar bases are satisfactory for growing *H. pylori*, e.g., brain heart infusion agar, Columbia agar. Concerning the growth supplement, it is mandatory to add blood or serum, which includes numerous nutrients (vitamins and oligoelements, etc.) which enhance *H. pylori* growth.

The proportion of blood or serum can be 5%, 7%, or preferably, 10%. Red blood cells can be lysed for these growth substances to be more readily available. Animal blood, e.g., sheep or horse blood, can be added. Other growth supplements such as starch\textsuperscript{13}, bovine serum albumin\textsuperscript{46} and Cyclodextrins, which are cyclic oligosaccharides produced from starch by enzymatic treatment retaining the same properties as starch, are employed\textsuperscript{86}.

Cellini et al, proposed a blood-free medium supplemented with isovitalex (2%) and hemin (10 mg/liter). They also added urea (20 g/liter) and a pH indicator (phenol red) to identify the urease-positive colonies\textsuperscript{15}. *H. pylori* grow best at a slightly acidic pH (5 to 6), in agreement with its ecological niche, the mucus layer, where a pH gradient exists.

Another supplement which may be helpful to readily identify *H. pylori* colonies is 2, 3, 5-triphenyltetrazolium chloride (40 mg/liter). This compound is reduced by *H. pylori* to insoluble red formazan complexes, resulting in easily distinguished pigmented golden colonies.
Different selective supplements containing antimicrobial compounds have been proposed: vancomycin or teicoplanin to inhibit gram-positive cocci; polymyxin, nalidixic acid, colistin, trimethoprim, or cefsulodin to inhibit gram-negative rods; and nystatin or amphotericin B to inhibit fungi.

Non selective media such as Chocolate agar, Brain heart infusion agar with 5% horse blood, Brucella agar with 5% Sheep blood and Tryptone Soya agar with 5% sheep blood can be used. Selective media include Skirrows Campylobacter Medium and Brain heart infusion agar with vancomycin [6ug/ml] nalidixic acid [20ug/ml] and amphotericin [2ug/ml] have given good recovery.

Several studies performed in the early days of H. pylori detection showed the importance of using both a nonselective medium and a selective medium.

A critical point is to use fresh media (less than a week old) which is kept in closed boxes at 4°C to maintain humidity and avoid light exposure. Helicobacters are microaerophilic and capnophilic. Several systems can be used to achieve a microaerobic atmosphere, from the most sophisticated systems, such as a microaerobic cabinet or an incubator with an adjustable gas level, to jars in which the adequate atmosphere is created with an automatic apparatus or with H2-C02-generating packs.

The atmosphere in jars will vary according to the quantity of bacteria consuming oxygen; therefore, the gas pack should be changed every other day.
While *H. pylori* growth is possible in a candle jar\(^2\), it takes a longer time and results in small colonies.

The optimal culture temperature is 37°C, testifying to the adaptation of this bacterium to humans. For primary culture under optimal conditions, colonies may appear after 3 days and are at their optimum on day 4. However, in the case of negative culture, a 7- to 10-day incubation is recommended to ensure that the result is negative; if only a few organisms are present, this time lapse may be necessary to visualize the colonies.

In contrast, subcultures only take 2 to 3 days. When few colonies are present, the recommendation is to subculture by plating the colonies on a small area of the agar plate. It is important to remember that once *H. pylori* reaches its growth plateau, it becomes coccoidal and loses its viability, most likely due to a lack of adequate nutrients.

**Broth culture.**

Brain heart infusion or Brucella Broth with 1-10% fetal calf serum\(^5\) may be preferable for studies on physiology and metabolism.

**Identification of Helicobacter pylori in culture\(^1\)**

The growth of small, circular, smooth grey and translucent colonies observed after 3 to 4 days on the selective media plated with gastric biopsy specimens is an important criterion for *H. pylori* identification.
Gram staining of the colonies reveals gram negative curved rods, the spiral forms being less obvious. The characteristic gull wing is seen in broth cultures. Motility is best demonstrated in broth cultures and is weak when grown on agar.

The identification of culture consists essentially of testing for the presence of certain enzymes: cytochrome oxidase, catalase, and urease which are positive.

The ApiCampy strip\textsuperscript{59} identification of \textit{H. pylori} via positive urease, glutamyl transpeptidase, and alkaline phosphatase and negative nitrate reductase and hippuricase. Its resistance to nalidixic acid and sensitivity to the antibiotic cephalothin, helps in distinguishing it from other species.

**Histopathological Diagnosis**\textsuperscript{1}

\textit{H.pylori} can be identified with haemotoxylin and eosin but the bacteria can be more reliably seen with special stains\textsuperscript{1} such as acridine orange, modified Giemsa, cresyl violet or warthin-starry stains.

The typical morphology of \textit{H.pylori} is a comma shaped bacillus observed on the epithelial surface.

Gram staining of the touch smear of the biopsy specimen by rubbing it forcefully on a glass slide was used to confirm the presence of \textit{Campylobacter pylori} by Montgomery et al 1988\textsuperscript{77} this method had a sensitivity and specificity of 92% and 100% respectively.
Nijhawan et al.\textsuperscript{82} used the gastric crush cytology in the detection of \textit{H. pylori} infection and highlighted the advantage of crush smears.

Warthin - Starry silver stain demonstrates \textit{H. pylori} clearly as spiraled black rods against a yellow background. In Giemsa - stained sections, the organisms are clearly visible as Giemsa - positive (dark blue) spiraled rods.

M. Anjana et al.\textsuperscript{8}, evaluated the staining method of impression smear by Gram, two-step Gram, dilute carbol-fuchsin and Giemsa.

**Urease Tests**

The discovery that \textit{H. pylori} are a strong urease producer was made by Langenberg et al.\textsuperscript{55} and was used for rapid diagnosis by McNulty and Wise\textsuperscript{71}. When a biopsy specimen containing \textit{H. pylori} is introduced into a urea-rich medium, the urease hydrolyses the urea down into carbon dioxide and ammonia. The ammonium ion increases the pH, and a pH indicator, e.g., phenol red, changes color, in this case from yellow to red.

Modifications include McNulty and Dent\textsuperscript{72} buffered 40\% urea solution and Hazell’s\textsuperscript{37} solution with a high concentration of urea and pH indicator. Standardization of urease test was studied by Vinci. S. Jones et al. The various factors such as the concentration of urea in broth need for buffering the solution and addition of antibiotics were investigated\textsuperscript{117}.

A.V.Thillainayagam et al described the use of an ultra rapid endoscopy room test in which unbuffered urea solution with indicator was used. The test had a sensitivity and specificity of 89\% and 100\%.\textsuperscript{110}
Commercial Kits

The first-generation commercial kits were agar based, e.g., the CLO test. The new generation kits introduced in 1995 are strip-based tests.

In the first study, Rogge et al \(^9\), compared this new test to the CLO test which showed 99% sensitivity and 95% specificity after 2 h, which is superior to those of the CLO test.

Polymerase chain reaction\(^3\) [PCR]

The PCR was developed in the 1980s and thereafter quickly applied to the detection of *H. pylori*. Its application in the field of *H. pylori* concerns not only the detection of the bacterium but also its quantification and detection of specific genes relevant to pathogenesis (*CagA*) and specific mutations associated with antimicrobial resistance.

The first targets used were the genes of the urease operon: *ureA* and *glmM*, or the 16S rRNA gene.

Two main pathogenic factors the *Cag* PAI and the polymorphism of the *VacA* gene and other genes involved in adherence (*babA2*, *sabA*) or in pathogenicity (*oipA*, *dupA*, *iceA*) can also be detected by PCR. The new real time PCR technique is considered a breakthrough as it allows quantification and detection of point mutation associated with antibiotic resistance.
NON- INVASIVE TEST

The first method used was serology. However, due to the difficulty in obtaining an optimal specificity, other methods have been proposed namely Urea breath test, stool antigen test, and most recently, detection of specific antibodies in urine or saliva.

Urea Breath Test [UBT] ^66

A solution of labeled urea ingested by the patient is rapidly hydrolyzed by *H. pylori* urease, the labeled CO₂ is absorbed by the blood and exhaled in expired air. If the patient is not infected, most of the isotope is eliminated in urine without modification.

When [13C] urea is used, a specimen collection is performed before and 30 min after the ingestion.

The 13C/12C ratio is measured in both specimens, and the result is expressed as the difference between the two measurements. The need for a baseline value is due to the various amounts of 13C present in breath according to one's diet. When [14C] urea is used, specimen collection occurs only 20 min after ingestion.

Stool Tests

*H. pylori* culture from stools is not used as a routine diagnostic method. The first report of successful detection of *H. pylori* antigens in stools was made in 1997 by Kozak et al^48^ who reported an enzyme-linked immunosorbent assay
(ELISA) performed on stools this test was named *H. pylori* stool antigen test (HpSA).

**Serodiagnosis**

*H. pylori* infection is a chronic condition and immunoglobulin G (IgG) (subclasses 1 and 4) is the predominant immunoglobulin class, even in children. IgG are present at the mucosal level and detected in virtually all blood samples.

IgM are rarely observed, merely because acute *H. pylori* infections are seldom available for study.

In the experimental infection carried out by Morris et al\textsuperscript{78}, an initial IgM response was observed. IgA are also elevated in the majority of infected cases but not in all. Therefore, as the relevance of IgM and IgA is limited, commercial kits are primarily designed to detect IgG.

A complement fixation test was the first to be used in 1984 with 85% sensitivity\textsuperscript{41}, but currently the standard ELISA and its derivatives, such as rapid immunoenzymatic assays and immunoblotting are being used.

The performance of an ELISA is largely dependent on the nature of the antigens used. The first antigens to be used were whole-cell sonicates. A partial purification to obtain surface antigens can be achieved by glycine acid extraction. ELISA’s with these complex antigens had a sensitivity of 95% with a specificity of 90% according to Newell and Rathbone\textsuperscript{84}. Serological tests to detect *CagA* antibodies have also been designed based on recombinant proteins.
False-negative result may occur following a new infection before the antibody level is sufficiently elevated there also appears to be an increased prevalence of false negatives in the elderly. The very slow decrease in antibodies after eradication (25% in titer within 6 months or more) is also a cause of a false-positive result.

For this reason, the tests detecting active infection are preferable. Recent data have shown that serology is the best method in difficult situations.

Where bacterial density may be low due to gastric atrophy or due to previous treatment with Proton pump inhibitors or antibiotics, as is now frequently the case.

**Development of point-of-care tests**

They are essentially based on the diffusion of antibodies from a drop of serum or whole blood obtained by finger puncture through a membrane and an immunoenzymatic reaction. The first test proposed had a very promising performance (sensitivity, 92%: specificity, 88%). However the point-of-care tests have not been recommended in the Consensus Conferences.

**Immunoblot analysis and detection of CagA antibodies.**

The immunoblot is most likely used as a second-step technique to identify false-positive cases detected by ELISA, in which case, the criteria proposed by Nilsson et al must be used. A commercial immunoblot test is now available, which is an important advance towards standardization.
Detection of *H. pylori* antibodies in urine.

Specific *H. pylori* IgG antibodies are eliminated in urine but at very low concentrations. Alemohammad et al\(^6\) presented the first data in 1993 using both ELISA and immunoblotting with good accuracy.

**Detection of *H. pylori* antibodies in saliva**

Detection of IgG antibodies is used by ELISA or by immunoblotting. Patel et al studied efficacy of various kits\[^{89}\].

**Treatment\(^{39}\)**

The current recommended treatment for *H. pylori* eradication includes two antibiotics and an antisecretory drug, essentially a PPI, to which a bismuth salt can be added. The most commonly used association worldwide is a double dose of PPI (omeprazole, lansoprazole, pantoprazole, rabeprazole, or esomeprazole) plus clarithromycin (500 mg twice a day [b.i.d.]) and amoxicillin (1 g b.i.d.) for 7 days (treatment 1). Other 7-day regimens include a double dose of PPI plus clarithromycin (500 mg b.i.d.) and metronidazole (500 mg b.i.d.) (Treatment 2) or a double dose of PPI plus amoxicillin (1 g b.i.d.) and metronidazole (500 mg b.i.d.) (Treatment 3), with the latter being mostly used as a second line treatment for 14 days in the case of failure of treatment 1.
The Antimicrobial Susceptibility

Like any infectious agent\textsuperscript{59}, \textit{H. pylori} can acquire resistance to antimicrobial agents used to treat the infection, and therefore, susceptibility testing is important in the management of the infection.

\textit{H. pylori} is intrinsically resistant to glycopeptides, cefsulodin, polymyxins, nalidixic acid, trimethoprim, sulfonamides, nystatin, amphotericin B, and cycloheximide. Some of these are used as selective agents in isolation media. \textit{H. pylori} acquire resistance by mutation.

Susceptibility Testing Methods

1. Phenotypic method

\textit{(a) Agar dilution method}: The agar dilution method, usually considered the reference method to compare other techniques, has been proposed by the Clinical Laboratory Standard Institute (CLSI) as the method to be used for \textit{H. pylori} clarithromycin susceptibility testing\textsuperscript{80}.

\textit{(b) Broth dilution method}: It has rarely been used for \textit{H. pylori} because of the difficulty of growing, this bacterium in broth\textsuperscript{18}.

\textit{(c) Disk diffusion testing}: The disk diffusion method is the simplest and most economic for susceptibility testing.

\textit{(d) Etest}: The latest method has the advantage of being a quantitative method with a direct expression of MICs, and furthermore, it is adapted to slow-growing bacteria like \textit{H. pylori}. 

29
**Genotypic detection of resistance.**

*H. pylori* resistance is essentially due to chromosomal mutations which can be easily detected with molecular tests. Resistance to macrolides, fluroquinolones, tetracycline and metronidazole can be detected by RT-PCR. Flurosent-in-situ-hybridization also has been used to detect clarithromycin resistance⁴².

**VACCINES⁴**

First evidence that protective immunization may be a possibility came from Czinn and Nedrud in 1991. Ureas e was the early favorite vaccine candidate. Later both *HspA* and *HspB* protein were also shown to be immunogenic. Because of this and their apparent surface location, these proteins appeared as likely vaccine candidates. *CagA* and *VacA* are also being studied as vaccine candidates. Vaccine trails has been successful in animal models and studies are going on for a safe and effective vaccine.
AIMS OF THE STUDY

1. Detection of *Helicobacter pylori* in outpatients and inpatients presenting with upper Gastrointestinal symptoms.

2. To compare the various tests like Gram stain, Geimsa stain, Rapid urease, Culture and Histopathology for the identification of *H.pylori*.

3. To find out the PCR positivity in gastric biopsy samples.

4. To evaluate the antibody response to *H.pylori* by Immunochromatography test and ELISA method.

5. To correlate the *H.pylori* infection with Endoscopic clinical diagnosis.
MATERIAL AND METHODS

ETHICAL CONSIDERATION

The study was conducted with the approval from the institutional Ethical Committee Government General Hospital and Madras Medical College, Chennai-3. Permission to conduct the study was sought from the respective hospital authorities. Informed consent was obtained from the patients before the enrolment into the study.

PERIOD OF STUDY

This is a prospective cross sectional study conducted over a period of one year from April 2008 – April 2009.

PLACE OF STUDY

This study was carried out at the Institute of Microbiology, Madras Medical College and Research Institute in collaboration with the Department of Medical Gastroenterology, Government General Hospital, Chennai.

STUDY GROUP

Outpatients and inpatients, of both sexes in the age group 20-69 years, based on the following criteria were included in the study.
Inclusion criteria:

- Patients with complaints suggestive of upper gastrointestinal diseases.
- Patients with gastric ulcer, duodenal ulcer, antral gastritis and gastric carcinoma.
- Patients who were not on antibiotics, proton pump inhibitor or Helicobacter eradication therapy within 1 month prior to inclusion in this study.

Exclusion criteria:

- Patients with previous gastric surgery.
- Patients with active bleeding.

STUDY DESIGN

The details of complete history, clinical features of the patients to be subjected to endoscopy were obtained. Preinvasive procedure preparation for Oesophago-gastro-duodenoscopy was performed as per norms. Biopsy tissue was collected from the gastric antrum of the patient and the specimens were submitted for. Histopathological study, gram stain, culture, rapid urease test and PCR for identification of *H pylori* infection. A patient with *Helicobacter pylori* infection was defined as those patients who were positive for at least two out of the three evaluation tests. [Montgomery et al198877]; [Sengupta.S 2002-92103]
SPECIMEN COLLECTION AND TRANSPORT

Biopsy Sample

Patients fasted overnight before endoscopy. Endoscopy was done using fiber optic endoscope. The endoscope and the biopsy forceps were rinsed thoroughly with water and soaked in 2% gluteraldehyde for 20 minutes\textsuperscript{17} and were thoroughly rinsed with sterile normal saline just before the collection of specimen.

Five biopsy samples were taken from the antrum (2cm from the pylorus) and were transferred to respective Eppendorf tube under sterile conditions. One sample was inoculated into urea broth for rapid urease, two specimens were transported in normal saline for culture, Gram stain and Giemsa stain, one in phosphate buffer saline for PCR and last specimen was placed in 10% formalin for histopathological examinations. The specimens for PCR were stored in phosphate buffer saline at -70°C.

The specimens for culture were transported in ice to the laboratory and were inoculated on the culture media without delay.

Blood

3ml of venous blood was collected under aseptic precautions; serum was separated and stored at -20°C for further processing.
PROCESSING OF SPECIMENS

Rapid Urease Test

An antral biopsy tissue was placed in an Eppendorf tube containing 0.5 ml of freshly prepared 8% urea at a pH of 6.8 to which had been added two drops of 1% phenol red as a pH indicator. Colour change from yellow to red at room temperature within two hours, were taken as positive [Raul et al 2004].

Culture

Biopsy tissue was crushed between two sterile glass slides and the minced tissue was inoculated onto freshly prepared campylobacter agar base with 5% defibrinated sheep blood and Skirrows supplement (selective media) and chocolate agar (non selective media). The plates were incubated at 37°C in a candle jar with a pad of cotton soaked in water placed at the bottom. The plates were examined for bacterial growth between three to seven days. Characteristic small, translucent circular colonies were confirmed by gram stain, catalase, oxidase and urease. They were sub cultured onto chocolate agar and campylobacter agar with skirrows supplement (Vancomycin 10 mg, PolymycinB 2500IU and Trimethoprim 5mg) till no growth was obtained.

Confirmatory tests for suspected colonies

1. **Gram stain**-Gram negative curved bacilli were seen.

2. **Oxidase test**-The suspected colony was streaked on the surface of oxidase strip containing 1% tetramethyl paraphenylene diamine dihydrochloride. An intense purple colour developed within 5 seconds and was recorded as positive. Positive and negative controls were used.
3. **Urease test** - The colony was emulsified in 0.5 ml of the urea broth. An instant colour change from yellow to pink was noted as positive.

4. **Catalase test** - The suspected colony was introduced with a glass rod into 3% Hydrogen peroxide taken in a clean test tube. Immediate production of gas bubbles was noted as positive. Positive and negative controls were also tested.

**Crush cytology**

Another biopsy tissue was crushed between two sterile glass slides and the minced tissue was used to make two smears.

**Gram stain**

One of the slides was air dried and heat fixed. The slide was covered with methyl violet for one minute, excess stain was poured off, Grams iodine was added and washed after 1 minute. This was followed by acetone for 2-3 seconds. The acetone was washed and the slide was counter stained with dilute carbol fuschin for one minute, washed with water, blotted dry and observed under oil immersion objective. *Helicobacter pylori* appeared as gram negative curved bacilli.

**Giemsa stain**

The other slide was air dried and fixed with methanol for 3 minutes, 2-3 drops of undiluted Giemsa stain was added and kept for 5 minutes. The smear was then washed with water, blotted dry and seen under oil immersion
objective. The organism appeared deep purple with the typical gull-wing morphology.

**Histopathology**

One specimen was fixed in 10% formalin, paraffin sections were made and stained with Haematoxylin and Eosin and examined for *Helicobacter pylori*.

**Serology**

The serological detection of IgG antibodies to cellular components of *Helicobacter pylori* was done using **EURO IMMUN ELISA**. The antigen coated in the wells are provided by the strain Lior 1 of Helicobacter pylori. The cultured bacteria have been disrupted in alkaline buffer. The antigen mixture used contains all significant proteins as verified by SDS polyacrylamide gel electrophoresis.

Serology was done for 130 cases with gastro duodenal symptoms, 1 positive control, 1 negative control and 3 calibrated standards were provided by the manufacturer.

**Methods**

Serum samples were diluted 1:101 before assay (10ul of serum was diluted with 1ml of sample diluent). 100ul of each calibrated standard or diluted sample was dispensed into the wells. The plate was incubated for 30 minutes at room temperature. The wells were washed thoroughly thrice using
wash buffer. The micro plate was blotted on absorbent paper. 100ul conjugate consisting of rabbit anti human IgG conjugated with Horse radish peroxidase was added immediately into each well. The plate was incubated for 30 minutes at room temperature.

Following incubation, the plate was washed 3 times with wash buffer. 100ul of chromogen/substrate solution was added into each well. The plate was incubated for 15 minutes. Blue colour developed in the wells. 100ul of stop solution was added to each well in the same order as the chromogen substrate to allow equal reaction times. Blue colour changed to yellow on addition of the stop solution. The optical density was read at 450 nm in a microplate reader within 30 minutes of adding the stop solution.

**Calculation of results**

The optical density of each calibrator was plotted against its concentration and a curve was drawn through the points. The unknowns were read off the curve.

**Interpretation of results as per kit recommendations**

Values below 16 Ru/ml-negative.

Values between16-22Ru/ml-intermediate.

Values above 22Ru/ml-positive.
One step antibodies to *H. pylori* test by SDBIOLINE *H.pylori* kit.

The SDBIOLINE *H. pylori* test contains a membrane strip, which is pre-coated with *H. pylori* capture antigen on test band region. The *H. pylori* antigen colloid gold conjugate and serum sample moves along the membrane chromatographically to the test region (T) and forms a visible line as the antigen–antibody-antigen gold particle complex forms with high degree of sensitivity and specificity.

The SDBIOLINE *H. pylori* test is a rapid test for the qualitative detection of antibodies of all isotypes (IgG, IgM, IgA, etc) specific to *Helicobacter pylori* in human serum, plasma or whole blood.

Procedure

1. Remove the test device from the foil, and place it on a flat, dry surface.

2. Transfer 10 µl of serum or plasma. Add 3 drops of assay diluent (approximately 110 µl) and start the timer.

3. Interpret test results at 10 minutes. The result should not be interpreted after 10 minutes.

Interpretation of the test:

1. **Negative Result:** The presence of only one purple colour band within the result window indicates a negative result.
2. **Positive Result:** The presence of only two colour bands (“T” band and “C” band) within the result window, no matter which band appears first, indicates a positive result.

3. **Invalid Result:** If the purple colour band is not visible within the result window after performing the test, the result is considered invalid.

**DIAGNOSIS OF *H. pylori* INFECTION BY PCR.**

In this study PCR was used to detect Helicobacter. Pylori by using primers specific for 16s rRNA gene. This gene is a highly specific target for amplification and has been previously used for reclassification of the organism\(^\text{98}\). \(16s \text{ rRNA}\) is targeted to confirm *H.pylori* infection and positive amplification of *H.pylori* specific DNA may be considered as a direct evidence of the presence of the pathogen.\(^\text{100}\)

**Preparation of samples for PCR amplification:**

50 gastric biopsy samples were chosen for PCR study. 50% of culture positive samples (11 out of 23) and 39 culture negative samples were processed for PCR detection for 16S rRna of *H.pylori*. The sample is homogenized in the DNA extraction solution [GeNei] TM. The genomic DNA is precipitated from the lysate with ethanol. Following an ethanol wash, DNA is solubilized in \(1\times \text{TE BUFFER}\).
The primer was derived from the region of the 16S rRNA.

Forward primer- 5’GCTAAGAGATCAGCTATGTCC3’

Reverse primer- 5’TGGCAATCAGCGTCAGGTAATG3’

**PCR Reaction Mixture Components:**

<table>
<thead>
<tr>
<th>Components</th>
<th>Final Concentration of reagents</th>
<th>Quantity of reagents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Template DNA</td>
<td>5-100 ng</td>
<td>1 μL</td>
</tr>
<tr>
<td>dNTP 2.5mM</td>
<td></td>
<td>1 μL</td>
</tr>
<tr>
<td>Buffer (10x)</td>
<td>1x</td>
<td>5 μL</td>
</tr>
<tr>
<td>F.R 20 pimol</td>
<td>0.4 μm</td>
<td>1 μL</td>
</tr>
<tr>
<td>R.P 20 pimol</td>
<td>0.4 μm</td>
<td>1 μL</td>
</tr>
<tr>
<td>Taq.Poly</td>
<td>2 μm</td>
<td>0.2 μL</td>
</tr>
<tr>
<td>MgCl2</td>
<td></td>
<td>2.5 mM</td>
</tr>
<tr>
<td>D.W</td>
<td></td>
<td>40.3 μL</td>
</tr>
<tr>
<td>Total vol.</td>
<td></td>
<td>50 μL</td>
</tr>
</tbody>
</table>

The PCR tubes are mixed well and are kept in the thermo cycler and the target DNA were amplified as given in the table below.
PCR parameters:

<table>
<thead>
<tr>
<th>Operation</th>
<th>Temperature</th>
<th>Time in mins</th>
<th>Cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Denaturation</td>
<td>95°C</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94°C</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>55°C</td>
<td>1</td>
<td>39</td>
</tr>
<tr>
<td>Elongation</td>
<td>72°C</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Final Elongation</td>
<td>72°C</td>
<td>7</td>
<td></td>
</tr>
</tbody>
</table>

The amplified PCR products were stored at 4° C until electrophoresis. Amplified products were run using horizontal 1.5% agarose gel electrophoresis. The gel was visualized using a UV trans illuminator. The amplified PCR products and 100 base pair DNA molecular markers were seen as bright fluorescent bands.

**Interpretation:**

A 500 bp corresponds to 16S rRNA genes specific oligo-nucleotides.
RESULTS

Table -1
Demographic Profile of Study Population

<table>
<thead>
<tr>
<th>AGE</th>
<th>MALE</th>
<th>FEMALE</th>
<th>TOTAL</th>
<th>PERCENTAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>20-29</td>
<td>7</td>
<td>2</td>
<td>9</td>
<td>6.9</td>
</tr>
<tr>
<td>30-39</td>
<td>23</td>
<td>8</td>
<td>31</td>
<td>23.8</td>
</tr>
<tr>
<td>40-49</td>
<td>32</td>
<td>12</td>
<td>44</td>
<td>33.8</td>
</tr>
<tr>
<td>50-59</td>
<td>26</td>
<td>7</td>
<td>33</td>
<td>25.3</td>
</tr>
<tr>
<td>&gt;60</td>
<td>10</td>
<td>3</td>
<td>13</td>
<td>10</td>
</tr>
<tr>
<td>TOTAL</td>
<td>98</td>
<td>32</td>
<td>130</td>
<td>100</td>
</tr>
</tbody>
</table>

Out of a total of 130 cases 98 were male (75.38%) and 32 were female (24.62%).

Chart -1

Demographic Profile of Study Population
### Table -2
Symptoms and Sex Distribution In Relation To Clinical Diagnosis

<table>
<thead>
<tr>
<th>SYMPTOMS</th>
<th>Gastric Carcinoma (n=19)</th>
<th>Acid-peptic disease (n=111)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>Epigastric pain</td>
<td>14</td>
<td>2</td>
</tr>
<tr>
<td>Vomiting</td>
<td>11</td>
<td>2</td>
</tr>
<tr>
<td>Dyspepsia</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>Loss of appetite</td>
<td>12</td>
<td>2</td>
</tr>
<tr>
<td>Weight loss</td>
<td>11</td>
<td>3</td>
</tr>
<tr>
<td>Haematemesis</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>Malena</td>
<td>5</td>
<td>1</td>
</tr>
</tbody>
</table>

Epigastric pain was the predominant symptom among patients with both acid peptic disease and gastric carcinoma.

### Chart -2

![Chart showing symptoms and sex distribution in relation to clinical diagnosis](image-url)
Table -3
Categorization of the study population based on Endoscopic diagnosis

<table>
<thead>
<tr>
<th>Endoscopic diagnosis</th>
<th>Total</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duodenal Ulcer</td>
<td>49</td>
<td>38%</td>
</tr>
<tr>
<td>Gastritis</td>
<td>44</td>
<td>34%</td>
</tr>
<tr>
<td>Gastric ulcer</td>
<td>18</td>
<td>14%</td>
</tr>
<tr>
<td>Gastric Carcinoma</td>
<td>19</td>
<td>15%</td>
</tr>
<tr>
<td>Total</td>
<td>130</td>
<td>100%</td>
</tr>
</tbody>
</table>

Shows that 49 patients had duodenal ulcer, 44 cases had gastritis, 18 cases had gastric ulcer and 19 cases had gastric carcinoma.

Chart - 3
Table – 4
Rapid Urease Positivity vs Endoscopic diagnosis

<table>
<thead>
<tr>
<th>Endoscopic diagnosis</th>
<th>Total</th>
<th>Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duodenal Ulcer</td>
<td>49</td>
<td>23</td>
</tr>
<tr>
<td>Gastritis</td>
<td>44</td>
<td>20</td>
</tr>
<tr>
<td>Gastric ulcer</td>
<td>18</td>
<td>4</td>
</tr>
<tr>
<td>Gastric Carcinoma</td>
<td>19</td>
<td>6</td>
</tr>
<tr>
<td>Total</td>
<td>130</td>
<td>53</td>
</tr>
</tbody>
</table>

41% of the cases (n=130) were positive by rapid urease test.

Chart - 4
Table -5

Time taken for Rapid Urease to become positive

<table>
<thead>
<tr>
<th>Time taken in minutes</th>
<th>No. of samples positive</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-5</td>
<td>21</td>
<td>40%</td>
</tr>
<tr>
<td>5-10</td>
<td>13</td>
<td>25%</td>
</tr>
<tr>
<td>10-15</td>
<td>10</td>
<td>19%</td>
</tr>
<tr>
<td>15-20</td>
<td>7</td>
<td>13%</td>
</tr>
<tr>
<td>20-120</td>
<td>2</td>
<td>4%</td>
</tr>
<tr>
<td>Total</td>
<td>53</td>
<td>41%</td>
</tr>
</tbody>
</table>

Maximum colour change was detected in the first 5 minutes

Chart - 5
Table -6

Positivity of *H pylori* by Gram stain

<table>
<thead>
<tr>
<th>Endoscopic diagnosis</th>
<th>No of cases</th>
<th>Positive</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duodenal Ulcer</td>
<td>49</td>
<td>20</td>
<td>41%</td>
</tr>
<tr>
<td>Gastric Ulcer</td>
<td>18</td>
<td>3</td>
<td>17%</td>
</tr>
<tr>
<td>Gastritis</td>
<td>44</td>
<td>14</td>
<td>38%</td>
</tr>
<tr>
<td>Gastric Carcinoma</td>
<td>19</td>
<td>5</td>
<td>26%</td>
</tr>
<tr>
<td>Total</td>
<td>130</td>
<td>42</td>
<td>32%</td>
</tr>
</tbody>
</table>

32% of *H. pylori* infection was detected by Gram stain

Chart - 6
Table -7

Distribution of Giemsa positivity in the Endoscopic Diagnosis

<table>
<thead>
<tr>
<th>Endoscopic diagnosis</th>
<th>No of cases</th>
<th>Positive</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duodenal Ulcer</td>
<td>49</td>
<td>23</td>
<td>47%</td>
</tr>
<tr>
<td>Gastric Ulcer</td>
<td>18</td>
<td>4</td>
<td>22%</td>
</tr>
<tr>
<td>Gastritis</td>
<td>44</td>
<td>19</td>
<td>43%</td>
</tr>
<tr>
<td>Gastric Carcinoma</td>
<td>19</td>
<td>5</td>
<td>26%</td>
</tr>
<tr>
<td>Total</td>
<td>130</td>
<td>51</td>
<td>39%</td>
</tr>
</tbody>
</table>

Giemsa staining was positive in 40% of the cases.

Chart - 7

Distribution of Giemsa positivity in the Endoscopic Diagnosis
Table -8

Comparison of urease with *H pylori* positivity in Gram stain, Giemsa stain and Histopathology

<table>
<thead>
<tr>
<th>Clinical status</th>
<th>No of cases</th>
<th>RUT</th>
<th>Giemsa</th>
<th>Gram</th>
<th>HPE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duodenal Ulcer</td>
<td>49</td>
<td>23</td>
<td>23</td>
<td>20</td>
<td>12</td>
</tr>
<tr>
<td>Gastric Ulcer</td>
<td>18</td>
<td>4</td>
<td>4</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Gastritis</td>
<td>44</td>
<td>20</td>
<td>19</td>
<td>14</td>
<td>8</td>
</tr>
<tr>
<td>Gastric Carcinoma</td>
<td>19</td>
<td>6</td>
<td>5</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>130</td>
<td>53</td>
<td>51</td>
<td>42</td>
<td>23</td>
</tr>
</tbody>
</table>

Rapid urease and Giemsa gave comparable results

Chart - 8
**Table -9**

Detection of *H pylori* by culture

<table>
<thead>
<tr>
<th>Culture</th>
<th>Total no. cases</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>POSITIVE</td>
<td>23</td>
<td>18%</td>
</tr>
<tr>
<td>NEGATIVE</td>
<td>107</td>
<td>82%</td>
</tr>
<tr>
<td>TOTAL</td>
<td>130</td>
<td>100%</td>
</tr>
</tbody>
</table>

18% of the cases were positive by culture

**Chart - 9**
Table -10

ELISA reading with Endoscopic diagnosis

<table>
<thead>
<tr>
<th>Endoscopic diagnosis</th>
<th>No of cases</th>
<th>Range EU/ml</th>
<th>Positive</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duodenal Ulcer</td>
<td>49</td>
<td>12-198</td>
<td>44</td>
<td>90%</td>
</tr>
<tr>
<td>Gastric Ulcer</td>
<td>18</td>
<td>8-154</td>
<td>2</td>
<td>11%</td>
</tr>
<tr>
<td>Gastritis</td>
<td>44</td>
<td>10-186</td>
<td>31</td>
<td>70%</td>
</tr>
<tr>
<td>Gastric Carcinoma</td>
<td>19</td>
<td>6-152</td>
<td>5</td>
<td>26%</td>
</tr>
<tr>
<td>Total</td>
<td>130</td>
<td>-</td>
<td>82</td>
<td>63%</td>
</tr>
</tbody>
</table>

Seroprevalence in the study population was 63%

Chart - 10

ELISA reading with Endoscopic diagnosis

- Duodenal Ulcer: 90%
- Gastric Ulcer: 11%
- Gastritis: 70%
- Gastric Carcinoma: 26%
Table -11
Comparative evaluation of conventional methods and ELISA based IgG antibody detection

<table>
<thead>
<tr>
<th>Type of Test</th>
<th>Patients (n=130)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Percentage</td>
<td></td>
</tr>
<tr>
<td>Rapid Urease</td>
<td>53</td>
<td>41%</td>
<td></td>
</tr>
<tr>
<td>Giemsa Staining</td>
<td>51</td>
<td>39%</td>
<td></td>
</tr>
<tr>
<td>Gram Staining</td>
<td>41</td>
<td>32%</td>
<td></td>
</tr>
<tr>
<td>Culture</td>
<td>23</td>
<td>18%</td>
<td></td>
</tr>
<tr>
<td>Significant IgG titres (22u/mL)</td>
<td>82</td>
<td>63%</td>
<td></td>
</tr>
</tbody>
</table>

Serology detected more cases than conventional tests

Chart - 11

Comparative evaluation of conventional methods and ELISA based IgG antibody detection
Table -12

Correlation of Immunochromotography with ELISA

<table>
<thead>
<tr>
<th>Name of Test</th>
<th>Positive</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immunochromotography</td>
<td>79</td>
<td>61%</td>
</tr>
<tr>
<td>ELISA</td>
<td>82</td>
<td>63%</td>
</tr>
</tbody>
</table>

ELISA was positive 82 in cases where as immunochromotography was positive in 79 cases

Chart - 12
Table -13

PCR positivity (16S rRNA gene) in different types of Endoscopic diagnosis

<table>
<thead>
<tr>
<th>Endoscopic diagnosis</th>
<th>No. Of Cases</th>
<th>Positive</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duodenal Ulcer</td>
<td>27</td>
<td>19</td>
<td>70%</td>
</tr>
<tr>
<td>Gastric Ulcer</td>
<td>4</td>
<td>1</td>
<td>25%</td>
</tr>
<tr>
<td>Gastritis</td>
<td>17</td>
<td>11</td>
<td>65%</td>
</tr>
<tr>
<td>Gastric Carcinoma</td>
<td>2</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td>Total</td>
<td>50</td>
<td>31</td>
<td>62%</td>
</tr>
</tbody>
</table>

PCR was positive in 70% of the duodenal ulcer cases, 65% of gastritis cases, and 25% of gastric ulcer cases.

Chart - 13
Table -14
Correlation of PCR with Combination of Tests

<table>
<thead>
<tr>
<th>RUT</th>
<th>Gram stain</th>
<th>Giemsa stain</th>
<th>Culture</th>
<th>PCR</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>8</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>1</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>2</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>8</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>5</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>2</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>5</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>19</td>
</tr>
</tbody>
</table>

- PCR detected 5 cases which were negative by other conventional tests.
- 8 cases were positive by RUT, Gram stain, Giemsa stain, Culture and PCR.
- 1 case was positive by RUT, Gram stain, Culture and PCR.
- 2 cases were positive by RUT, Giemsa stain, Culture and PCR.
- 8 cases were positive by RUT, Giemsa stain and PCR.
- 5 cases were positive by RUT, Giemsa stain and PCR.
- 2 cases were positive by RUT, Gram stain and PCR.
- 5 cases were positive by PCR.
- 19 cases were negative.
Table -15
Correlation of PCR with Conventional test in 50 samples

<table>
<thead>
<tr>
<th>Test</th>
<th>Positive</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR</td>
<td>31</td>
<td>62%</td>
</tr>
<tr>
<td>Culture</td>
<td>11</td>
<td>22%</td>
</tr>
<tr>
<td>RUT</td>
<td>26</td>
<td>52%</td>
</tr>
<tr>
<td>Gram</td>
<td>19</td>
<td>38%</td>
</tr>
<tr>
<td>Giemsa</td>
<td>23</td>
<td>46%</td>
</tr>
</tbody>
</table>

PCR detected the maximum number of positive cases when compared to conventional tests.

Chart - 15
### Table -16

**Correlation of PCR with Serology**

<table>
<thead>
<tr>
<th>Name of Test</th>
<th>Positive</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR</td>
<td>31</td>
<td>62%</td>
</tr>
<tr>
<td>ELISA</td>
<td>32</td>
<td>64%</td>
</tr>
<tr>
<td>Immunochromotography</td>
<td>29</td>
<td>58%</td>
</tr>
</tbody>
</table>

ELISA was positive in 64% of cases, PCR in 62%, Immunochromotography in 29 cases

### Chart - 16

![Chart showing correlation of PCR with serology]
DISCUSSION

The present work is based on using conventional and molecular methods for detecting *Helicobacter pylori* infection. Four biopsy based tests namely rapid urease test, histopathological examination, bacterial culture and PCR and two serological tests were used. The conclusions from the study give fruitful thought about the relative merits and demerits of each method.

A total of 130 patients with upper gastrointestinal symptoms were enrolled in the study. Among them 98 (75.38%) were males and 32 (24.6%) were females. (Table -1).

The maximum number of patients in this study was in the age group 40-49. In the study conducted by D.Nair et al 79 out of the 136 patients, 116 were male and 20 were female is comparable to the present study which also showed males were more affected than females.

Among the patients with gastric carcinoma, epigastric pain was the presenting symptom in 84%, vomiting in 68%, loss of appetite and loss of weight in 74%, haematemesis in 37%, dyspepsia in 37% and malena accounted for 32%.

Among the patients with peptic ulcer disease the predominant symptom was epigastric pain in 87% of cases, dyspepsia in 80%, vomiting in 52%, loss of weight in 30%, loss of appetite 19%, haematemesis in 10% and malena in 5% of the cases.
Epigastric pain was the predominant symptom among patients with acid peptic disease and gastric carcinoma. (Table -2).

The endoscopic examination of the study population revealed that duodenal ulcer accounted for 38%, gastritis in 34%, gastric carcinoma in 15%, and gastric ulcer in 14%. (Table -3)

All of the 130 patients were subjected to Rapid urease test, Gram staining, Geimsa Staining, Histopathology, Culture and Serology. PCR was performed on 50 cases which included 27 patients with duodenal ulcer, 17 with gastritis, 2 with gastric carcinoma and 4 with gastric ulcer.

Out of the 130 samples studied by Rapid urease test, 53 (41%) were positive (Table -4). The overall positivity of RUT correlated well with reports by Sivaprakash et al (38.7%) \(^{104}\), While it was lower than that reported by Maimooma et al (65.8%) \(^{61}\). In the present study 96% of the cases were positive within the first 20 minutes (Table-5). This is comparable to 95% reported by Senguta et al \(^{103}\). Marshall et al using a CLO test reported that 75% of the positive tests are detected within 20 minutes, 92% at 3 hour and 98% at 24 hours \(^{64}\). The present study showed more rapid change probably due to omission of buffer.

The direct Gram stained smears in our study showed positivity in 32% which is comparable to the study by U.Arora et al\(^{9}\). While Anjana et al reported 72.3% \(^{8}\). Other studies report values, ranging between 44-74% \(^{61,77}\) (Table6).
In the present study Giemsa staining of the crushed smear was positive in 39% of the smears studied. This correlates with the findings of 31% by Philip.E.Coudron et al94. (Table- 7).

Histopathological examination by Haematoxylin and Eosin staining was positive in 23 cases whereas Giemsa showed 53 positive cases. Studies by Aarti et al1 and Madan et al 60 also report that Giemsa technique showed greater positivity than Haematoxylin and Eosin staining. (Table- 8).

In the present study rapid urease detected 53 positive cases, Giemsa staining 51 cases, Grams staining 41 cases and Haematoxylin and Eosin 23 cases. The present study shows that Giemsa and rapid urease tests are comparable.

A similar picture emerges in the study by Anjana et al, in which 34 out of 47 cases were urease positive, while Giemsa was positive in 38 out of 47 of the cases. Culture positivity in the present study was 17.6%. U.Arora et al reported culture positivity of 28%. This low isolation rate may be due to the patchy distribution of *H.pylori* in gastric mucosa, its fastidious nature, mucosal atrophy\(^9\), intestinal metaplasia, administration of antibiotics[ to some other infections] and proton pump inhibitors\(^39\). (Table-9)

The present study reveals that serology was positive in 82 out of the 130 samples tested (63%) This is comparable to the study by D.Nair79 who reported a positive result in 64.9%.The distribution of positive serology results as against endoscopic findings in the study population is as follows- duodenal ulcer 44 cases, gastric ulcer 2 cases, gastritis 3 cases 1 cases and gastric
carcinoma 5 cases. Hence there exists a positive correlation between duodenal ulcer and *H. pylori*, confirming previous reports\(^{33}\). (Table-10)

Serology showed a greater number of positive cases than the conventional tests which may be due to past infection. This is comparable to the study by U. Arora et al, who reported greater case detection by serology than by conventional tests. (Table-11) The patchy distribution of organism in the gastric mucosa may have resulted in a lower value for biopsy based test. Another factor could be the presence of gastric atrophy and intestinal metaplasia that are hostile to *H. pylori*\(^90\).

The number of samples positive by ELISA was 82 as against 79 by immunochromatography. (Table-12)

The study by R.J.F.Laheiji et al\(^{57}\) showed that the serology kits that measured all isotypes specific to *H. pylori* did not perform as well as those that measured only IgG. This is comparable with the present study which used ELISA that detected IgG antibody and immunochromatographic test which detected all isotypes specific to *H. pylori*.

Among the 50 samples subjected to PCR, it was positive in 31 cases. The distribution of positive cases among the clinical conditions is as follows - duodenal ulcer 27 cases, gastritis 17 cases, gastric ulcer 4 cases and gastric carcinoma 2 cases. (Table-13)
PCR was positive in 62%, RUT In 52%, giemsa staining in 46%, Grams staining in 38% and culture22%. (Table-15). Thus PCR detected the most number of cases.

PCR detected *H.pylori* in all 11 biopsy samples that were culture positive. Out of the 20 culture negative samples 15 were detected by conventional methods and PCR. *H.pylori* in 5 samples were detected only by PCR. These 5 samples may be accounted for by the high sensitivity of this method or may have contained low number of viable cells and hence could not have been detected by routine diagnostic tests. Alternatively, the PCR assay may have detected the controversial “resting form “of the bacterium\(^{16}\), a proposed phase into which the vegetative form of the bacterium might differentiate under conditions of environmental stress. Clayton et al in his study reported that 7 out of 23 samples positive by PCR were negative by conventional tests.

All cases detected by PCR were also seropositive except one sample was negative by PCR, but positive by serology. It is possible that in this type of patient the ELISA detects a long lived antibody response. To *H.pylori* antigens well after an active infection has ceased. This is comparable to the study conducted by Marten Hammaret et al\(^69\) in which three samples positive by serology were negative by PCR.
SUMMARY

1. The majority of cases, out of a study population of 130 patients, were in the age group of 40-49 years.

2. A preponderance of males was noted among the study population.

3. Epigastric pain was the most common symptom in both gastric carcinoma and acid peptic disease.

4. Duodenal ulcer was the commonest endoscopic finding observed during the course of the study.

5. Rapid urease test was positive in 41% of the samples, 64.15% of which were positive in the first 10 minutes.

6. Gram stain was positive in 32% of the cases.

7. Giemsa stain was positive in 39% of the cases.

8. More number of samples was found to be positive for H. pylori by Giemsa stain (39%) as compared to those prepared by Gram stain (32%)

9. Culture for H. pylori was positive in only 18% of the cases.

10. The Seroprevalence of the study population was 63%.

11. ELISA based IgG antibody detected more positive cases than the conventional methods. (63% vs. 41%)
12. ELISA test detected more positive cases than immunochromotography test. (63% vs. 60%)

13. PCR which was positive in 62% of the study population detected more number of positive cases than by conventional methods. (62% vs. 52%).

14. Serology was positive in 64% of the cases as compared to 62% which were positive by PCR.
CONCLUSION

- A preponderance of *H. pylori* infection was noted in patients with duodenal ulcer.

- The simple and inexpensive Rapid urea test detected the maximum number of positive cases among the conventional tests. Hence it is a valuable adjunct to endoscopy.

- ELISA detected a significant number of *H. pylori* cases as compared to conventional methods however; the levels of *H. pylori* specific antibodies must be evaluated in the normal population to establish ELISA as a definitive diagnostic tool.

- ELISA is preferred to rapid test for the detection of *H. pylori* when facilities for Elisa are available.

- Isolation of organisms is feasible only in reference laboratories.

- PCR amplification of *H. pylori* DNA sequences has the potential to be a highly sensitive method for the laboratory diagnosis of *H. pylori* infection.
PROFORMA

Name : Occupation : Age/Sex :
Income : Address : O.P/I.P. No. :
Unit : Date :

H/O. PRESENT ILLNESS

Epigastric Pain : Vomiting/Nausea : Dyspepsia :
Haematemesis : Malena : loss of weight :
Loss of appetite : Others :

PERSONAL HISTORY

Alcohol : Smoking : h/o drug intake :
Diet Habits : Previous h/o Gastric surgery :

GENERAL EXAMINATION

Consciousness : Built : Nourishment :
Pallor : Jaundice : Cyanosis :
Clubbing : Pedal Edema : Lymphadenopathy :
Pulse : BP : Respiratory Rate :

Temperature :

SYSTEMIC EXAMINATION

Abd : Other systems :

INVESTIGATIONS :

UGI Scopy : USG Abd :
Rapid Urease : Grams stain :
Giemsa stain : Histopathology :
Culture : Serology :
PCR :
APPENDIX

1. Urea Broth

**Stock Solution A** [1% phenol red solution (free acid)]

1ml of phenol red was dissolved in 32.5 ml of 0.1 mol/L sodium hydroxide and made upto 100 ml distilled water. The solution was autoclaved for 15 minutes at 121°C.

**Stock solution B** (8% urea solution)

8gm of urea was dissolved in 100 ml of sterile distilled water under sterile precautions. The pH of the solution was adjusted to 6.8, and dispensed in 0.5 mL aliquots in sterile vials.

Two drops of 1% phenol red was added to each vial containing 0.5mL of 8% urea solution.

2. Chocolate Agar

Peptone : 1.0 gm  
Meat Extract : 1.0 gm  
Sodium chloride : 0.5 gm  
Agar agar : 2 gm  
Distilled water : 100 mL  
Defibrinated sheep blood : 10%

3. Skirrow’s Campylobacter Medium

I. Campylobacter agar base : 39.5 gm  
II. Distilled water : 1 litre  
III. Skirrow’s supplement : 1 vial consisting of  
    Vancomycin : 10 mg
Polymyxin B : 2500 IU
Trimethoprim : 50 mL

Sheep blood was used as horse blood was not available.
Amphotericin B 5 mg/L and 10% defibrinated sheep blood was used.

4. Phosphate Buffered Saline [PBS]

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>grams/ litre</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium chloride</td>
<td>8.0</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>0.2</td>
</tr>
<tr>
<td>Disodium hydrogen phosphate</td>
<td>1.15</td>
</tr>
<tr>
<td>Potassium dihydrogen phosphate</td>
<td>0.2</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000ml</td>
</tr>
</tbody>
</table>

pH was adjusted to 7.4

The above ingredients were dissolved in sterile distilled water and then filtered using filter paper.
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