Patients and Methods
PATIENTS AND METHODS

This study was conducted in the Departments of Surgery and Microbiology in the Jawaharlal Institute of Postgraduate Medical Education and Research (JIPMER) Hospital, Pondicherry, India from September 1994 to September 2000. The time schedule for the study is shown in the following figure.
Informed consent was obtained from all patients for inclusion in the study.

PATIENTS:

A total of nine hundred and fifty five patients were included in the study. The subjects of study were in the following four groups:

GROUP I - ASYMPTOMATIC ADULTS AND CHILDREN (n=205):

Group IA (n=105) - Children (≤ 15 years of age)
Group IB (n=100) - Adults

GROUP II - UPPER GASTROINTESTINAL TRACT DISORDERS (n=458):

Group II A (n=166) - Duodenal ulcer
Group II B (n=53) - Gastric ulcer
Group II C (n=50) - Non-ulcer dyspepsia
Group II D (n=51) - Carcinoma stomach
Group II E (n=50) - Chronic gastritis
Group II F (n=58) - Erosive gastroduodenitis
Group II G (n=30) - Stomal ulcer

GROUP III - COMPLICATED DUODENAL ULCER OTHER THAN PERFORATED DUODENAL ULCER (n = 142):

Group III A (n=30) - Bleeding duodenal ulcer
Group III B (n=112) - Duodenal ulcer with gastric outlet obstruction (These patients also formed part of Group II A)
GROUP IV - PERFORATED DUODENAL ULCER (n=262):

Group IVA (n=202) - Perforated duodenal ulcer (Prospective)
Group IVB (n=60) - Perforated duodenal ulcer (Retrospective)

All patients and controls formed part of the prospective study except group IVB.

GROUP I - ASYMPTOMATIC ADULTS AND CHILDREN:

A total of two hundred and five patients admitted to the surgical wards without any gastrointestinal disorders were included in this group. They were subdivided into Group IA (Children below and upto 15 years of age) and Group IB (Adults). All patients over 15 years of age were taken as adults.

GROUP IA:

One hundred and five children were included in this group and were divided into three sub-groups according to their age, i.e. upto 5 years of age, 6 to 10 years of age and 11-15 years of age. Gastrointestinal disorders were ruled out in these children on clinical examination as per proforma (Annexure-I). It was considered unethical to subject these children to endoscopy for this purpose. \( H\ pylon \) status was determined by serology for anti-\( H\ pylon \) IgG antibodies.

GROUP IB:

There were one hundred patients in this group. A detailed history and clinical examination was carried out to rule out gastrointestinal disorders as per the proforma (Annexure-I). After informed consent, an upper gastrointestinal endoscopy
was done under topical anaesthesia in these patients to rule out asymptomatic peptic ulcers or other upper gastrointestinal disorders. The *H pylori* status was determined by the urease test, histology and serology. As the detection of *H pylori* was done by invasive tests, only hospital based controls were included. These asymptomatic adults were similar in age and gender to patients in the perforated duodenal ulcer group. Since strict age and gender matching was not done, logistic regression analysis was carried out to adjust for age and gender as variables wherever a significant difference was found. (The scheme of study in this group is shown in the next page).

GROUP II – UPPER GASTROINTESTINAL DISORDERS:

A total of four hundred and fifty eight patients comprised this group. They were subdivided into eight sub-groups according to the disorder.

In these patients the history and findings on detailed clinical examination were recorded as per Proforma (Annexure-II). The diagnosis of the disorder was confirmed by upper gastrointestinal endoscopy. *H pylori* status was determined by urease test, histology and serology. The prevalence and serological titres of *H pylori* in different upper gastrointestinal disorders was compared with controls.
GROUP I – ASYMPTOMATIC ADULTS AND CHILDREN

ASYMPTOMATIC ADULTS AND CHILDREN

GROUP IA (CHILDREN < 15 years)

SEROLOGY FOR H. PYLORI

PREVALENCE OF H. PYLORI

COMPARISON OF PREVALENCE WITH OTHER DISORDERS OF UPPER GASTROINTESTINAL TRACT

GROUP IB (ADULTS > 15 years)

UPPER GASTROINTESTINAL ENDOSCOPY TO RULE OUT ASYMPTOMATIC PEPTIC ULCERS OR OTHER UPPER GASTROINTESTINAL PATHOLOGY

UREASE TEST, HISTOLOGY, SEROLOGY FOR DIAGNOSIS OF H. PYLORI INFECTION
The word prevalence is used in this study to indicate the occurrence of a positive *H pylori* status and not in its epidemiological connotation. This is also the definition adopted by other workers in the field. The various sub-groups were as follows. The scheme of study in this group is shown in the following page.

GROUP IIA:

**Duodenal ulcer** – This group included both patients with active and healed duodenal ulcers with gastric outlet obstruction. A total of one hundred and sixty six patients comprised this group. An active duodenal ulcer was defined as mucosal ulceration (breach in the mucosa with apparent depth) of 5 mm or more in diameter in the duodenum [255]. Patients classified as duodenal ulcer were subdivided into the following three groups:

(i) Uncomplicated active duodenal ulcer (n=54)

(ii) Gastric outlet obstruction with active duodenal ulcer (n=65)

(iii) Gastric outlet obstruction without active duodenal ulcer (n=47)

The diagnosis of gastric outlet obstruction was established when the endoscope could not be negotiated into the second part of the duodenum inspite of using antispasmodic drugs (Hyosine-N-butyl bromide). Specific epidemiologic factors related to prevalence rate included size and number of ulcers, smoking, alcohol and analgesic (NSAID) abuse.
GROUP II – UPPER ALIMENTARY TRACT DISORDERS

UPPER ALIMENTARY TRACT DISORDERS

GROUP IIA DUODENAL ULCER

GROUP IIB GASTRIC ULCER

GROUP IIC NON-ULCER DYSPEPSIA

GROUP IID CARCINOMA OF THE STOMACH

GROUP III CHRONIC GASTRITIS

GROUP IIIA EROSIVE GASTRO-DUODENITIS

GROUP IIG STOMAL ULCER

UPPER G.I. ENDOSCOPY + HISTOLOGY FOR CONFIRMING DIAGNOSIS

UREASE TEST, HISTOLOGY, SEROLOGY FOR DIAGNOSIS OF H. PYLORI

PREVALENCE OF H. PYLORI

CORRELATION OF PREVALENCE IN “CONTROLS” WITH VARIOUS UPPER ALIMENTARY DISORDERS
A smoker was defined as one who smoked cigarettes on a daily basis and whose habit was well established; that is, patients indicated that they had smoked cigarettes during and before the past year [256-258]. Non-smokers were those who denied all forms of current and previous smoking. Ex-smokers who had given up cigarettes at least a year before were classified as non-smokers. Alcoholics were defined as those who consumed alcoholic drinks (wine, beer, whisky, cocktails and locally made liquor) on a regular basis during and before the past year [256]. NSAID use was defined as consumption of these drugs on regular basis for a period of one month or more within the six months prior to endoscopy [259].

GROUP IIIB:

*Gastric ulcer* – There were fifty three patients with benign gastric ulcer. Diagnosis was confirmed by endoscopic biopsy of the ulcer and histology. A mucosal ulceration of 5 mm or more in the stomach was defined as a gastric ulcer [255]. Patients with associated duodenal ulcers (Type II gastric ulcers) were also included in this group [260]. The prevalence rate of *H pylori* in patients with gastric ulcer was related to the number of ulcers, smoking, alcohol and analgesic abuse.

GROUP IIIC:

*Non-ulcer dyspepsia* – Fifty patients with NUD were studied. All patients with recurrent upper abdominal pain or discomfort lasting for more than a month with symptoms for 25% or more of the time comprised this group [157]. Relevant clinical,
biochemical, endoscopic and radiologic examinations were done to rule out other gastrointestinal disorders. A stool examination for parasites was done in all patients. Amoebic serology (Indirect hemagglutination test) was done to rule out amoebic colitis. Abdominal ultrasound was done to exclude biliary tract disorders. No abnormality was detected on upper gastrointestinal endoscopy in this group.

GROUP IID:

Carcinoma of the stomach – There were fifty one patients in this group. Diagnosis was confirmed by multiple endoscopic biopsies for histology from the lesion. In these patients, endoscopic biopsies for detection of \textit{H} \textit{pylori} were taken from the pyloric antrum wherever possible. In case of a pyloric antral carcinoma, biopsies were taken from the adjacent normal looking mucosa. The \textit{H} \textit{pylori} status was correlated with tumour site. Tumors were classified as (a) antral, (b) fundic, (c) body and (d) diffuse when they involved the whole stomach. When tumours extended to more than one site, they were classified according to the area of maximal involvement.

GROUP IIE:

Chronic gastritis – There were fifty patients in this group. All patients had chronic antral gastritis. Diagnosis of chronic gastritis was made by endoscopy when it showed congested gastric mucosa in the pyloric antrum. However, it is well known
that endoscopically congested mucosa may not show the characteristic features of chronic antral gastritis and histological abnormality may be present when endoscopic appearance is normal [261,262]. Biopsies were taken for histological confirmation. Histological diagnosis was made according to established criteria [263]. Patients who did not have characteristic histological features of chronic gastritis were excluded from this group. Patients with associated duodenal ulcer were excluded from this group.

GROUP IIIF:

_Erosive gastroduodenitis_ – There were fifty eight patients with erosive gastroduodenitis. Erosive gastroduodenitis was defined as superficial erosions in the stomach and/or duodenum less than 5 mm in size with or without adherent clots [255]. All these patients presented with hematemesis. Patients with associated gastric or duodenal ulcer and esophageal varices were excluded. Erosions were grouped into four sites, i.e. fundus, body, antrum and the duodenum according to site of maximal involvement. The _H pylori_ status in these patients was related to site of the erosions in the stomach, NSAID use, smoking and alcohol abuse.

GROUP IIG:

_Stomal ulcers_ – There were thirty patients who had stomal ulcers following truncal vagotomy and gastrojejunostomy (anterior 4, posterior 26) for chronic
duodenal ulcer. A stomal ulcer was defined as a lesion with mucosal disruption and yellowish floor present on the stoma of the gastrojejunostomy, or just distally on the efferent loop. Very rarely stomal ulcers may occur on the afferent loop of the jejunum. Patients were excluded from this group if there was an associated active ulcer in the duodenum.

GROUP III – COMPLICATED DUODENAL ULCER OTHER THAN PERFORATED DUODENAL ULCER:

In this group, history and findings on detailed clinical examination were recorded as per proforma (Annexure II). The scheme of study in this group is shown in the following page.

GROUP IIIA:

_Bleeding DU_ – Thirty patients hospitalized for upper gastrointestinal hemorrhage from duodenal ulcers with stigmata of recent hemorrhage seen at endoscopy were included. _H pylori_ status in these patients was related to NSAID use and size of the ulcer. _H pylori_ positivity status was also compared between bleeding duodenal ulcer, uncomplicated duodenal ulcer and controls.

Patients with bleeding DU with associated gastric ulcers or other gastroduodenal diseases were excluded.
GROUP III – COMPLICATED DUODENAL ULCER OTHER THAN PERFORATED DUODENAL ULCER

BLEEDING DUODENAL ULCER

DUODENAL ULCER WITH GASTRIC OUTLET OBSTRUCTION

UPPER GASTROINTESTINAL ENDOSCOPY FOR CONFIRMING DIAGNOSIS

UREASE TEST, HISTOLOGY, SEROLOGY FOR DIAGNOSIS OF H. PYLORI

PREVALENCE OF H. PYLORI

COMPARISON WITH NORMALS AND UNCOMPLICATED DUODENAL ULCER
GROUP IIIB:

*Duodenal ulcer with gastric outlet obstruction:* Of the one hundred and sixty six patients with duodenal ulcer in Group IIA, one hundred and twelve had gastric outlet obstruction. These were subdivided into two groups (I) gastric outlet obstruction with active ulcer (n=65) and (ii) gastric outlet obstruction without active ulcer (n=47). The *H pylori* status in patients with duodenal ulcer with and without gastric outlet obstruction was compared.

**Exclusion criteria:**

All patients in Group I, II and III, who had taken antibiotics, *H₂* blockers or proton pump inhibitors in the month preceding presentation were excluded as these are known to suppress *H pylori* and thus give false results in the tests for determining *H pylori* status.

**GROUP IVA – PERFORATED DUODENAL ULCER (PROSPECTIVE):**

A total of 202 patients with perforated duodenal ulcer treated by a Graham patch were included in the prospective group. Those patients who underwent immediate definitive surgery were excluded from this study. Relevant facts in the history and the findings on detailed clinical examination were noted as per proforma (Annexure-III). In all patients the operative procedure of simple closure was essentially the same.
SURGICAL PROCEDURE:

The abdomen was opened by an upper midline incision. If the patient had a pre-perforation duration of dyspeptic symptoms of over 3 months, it was considered as a chronic ulcer. Others were considered acute [149]. Operative evidence of chronicity such as induration of ulcer margins, scarring or thickening of the stomach wall were not taken into consideration as these are considered unreliable in differentiating acute from chronic ulcers [149]. The size of the perforation was noted at laparotomy. The perforation was closed by three transverse sutures along with an incontinuity omental transfer using 00 polyglactin 910 (Vicryl-Ethicon inc.). A thorough peritoneal lavage was given with normal saline. The abdomen was closed by the Smead-Jones technique leaving peritoneal drains [264].

Postoperatively, patients were put on ampicillin, gentamicin and metronidazole in appropriate doses for seven days. These patients were randomised by using a random number table and sealed envelope technique into two groups, i.e. Group IVA₁ and Group IVA₂ to receive either ranitidine or quadruple therapy respectively. The scheme of study is shown in the following page.

Patients in Group IVA₁ were administered ranitidine in a daily dose of 150 mg b.i.d. orally as soon as the patients were able to take oral fluids after the perforation closure. This was given for 4 weeks.
GROUP IVA – PROSPECTIVE STUDY OF PATIENTS WITH DUODENAL ULCER PERFORATION

DUODENAL ULCER PERFORATION

SIMPLE CLOSURE + ANTIBIOTICS FOR PERITONITIS*

SEROLOGY FOR H. PYLORI

RANDOMISE

GROUP IVA1 – RANITIDINE ALONE

GROUP IVA2 – QUADRUPLE THERAPY

REVIEW ENDOSCOPY AT 8 WEEKS UREASE, HISTOLOGY, SEROLOGY

NO ULCER

ULCER

RANITIDINE

REVIEW ENDOSCOPY AT 6 MONTHS UREASE, HISTOLOGY, SEROLOGY

NO ULCER

ULCER

RANITIDINE

REVIEW ENDOSCOPY AT 12 MONTHS UREASE, HISTOLOGY, SEROLOGY

VIEW ENDOSCOPY @ 6 MONTHLY INTERVALS FOR 2 YEARS UREASE, HISTOLOGY

OTHER DRUGS

NO RESPONSE

REFRACTORY ULCER

BLEEDING, GASTRIC OUTLET OBSTRUCTION

SURGERY

*AMPICILLIN 500 mg QID, GENTAMICIN 80 mg BID, METRONIDAZOLE 400 mg TID
Patients in Group IVA₂ were administered a quadruple antimicrobial adjuvant therapy for eradication of *H pylori* as soon as the patients were able to take oral fluids. The following drug schedule was followed:

- Tab. Ranitidine 150 BID – 4 weeks
- Tab. Colloidal Bismuth Subcitrate 120 mg QID – 2 weeks
- Tab. Metronidazole 400 mg TID – 2 weeks
- Cap. Tetracycline 500 mg QID – 2 weeks

The classical triple therapy was used as it was considered a gold standard and the first practical choice when the study was started if antibiotic sensitivity was not available [153,217-218].

All patients in Group IVA₁ and IVA₂ received metronidazole postoperatively, but patients in Group IVA2 received it for a period of 2 weeks as a component of ranitidine + “TRIPLE THERAPY” (Quadruple therapy). Complications of therapy were noted.

**FOLLOW UP:**

All patients were called for personal interview and detailed evaluation during the review and follow up. At endoscopy, mucosal ulceration of 5 mm or more in the duodenum was considered as persistent/recurrent ulceration [255]. Both the groups were reviewed as outpatients at 8 weeks, 6 months and 6 monthly thereafter. In
order to ensure compliance, drugs were issued to the patients for the complete period with detailed instructions for taking them. If there was a persistence of ulcer at the end of 8 weeks, both groups received ranitidine. Ulcer healing was defined as either complete re-epithelialization of duodenal mucosa or the presence of a scar [261]. At every review visit during the follow-up, urease test and histology was done on the endoscopic mucosal biopsies from the pyloric antrum and corpus to determine eradication of \textit{H pylori}. Two antral and two corpus biopsies were taken. Corpus biopsies are particularly valuable to assess \textit{H pylori} status following treatment [265,266]. Eradication was defined as a negative urease test with a negative histology for detection of \textit{H pylori}. The surgeon who performed endoscopy was not aware to which group the patient belonged.

The prevalence of \textit{H pylori} in patients with perforated duodenal ulcer was correlated with age, gender, size of ulcer, chronicity, smoking and alcohol abuse. The prevalence of \textit{H pylori} infection in the postoperative period was related to ulcer recurrence. A comparison of eradication rate between ranitidine and quadruple therapy was carried out at follow up.

During follow up if patients had any complications like gastric outlet obstruction, or refractory ulceration resistant to medical treatment they were referred for surgery. \textit{H pylori} serology for \textit{IgG} antibodies was done to assess the levels of \textit{IgG} antibodies at 6 months and 1 year review. The sensitivity, specificity, positive predictive value, negative predictive value and accuracy of using percentage fall in
serology as evidence of eradication in comparison to urease and histology as the gold standard was calculated using a decline of 10%, 20%, 25%, 30% and 50% in IgG titres (EU/ml). The stored serum at presentation, 6 months and 1 year were assayed concurrently (using same ELISA plate) to detect the level of antibodies. A comparison was made of fall in serological titres in eradicated and non-eradicated patients using different cut-off values (vide infra).

GROUP IVB – PERFORATED DUODENAL ULCER (RETROSPECTIVE):

Patients who were operated for perforated duodenal ulcer with the procedure of simple closure five or more years prior to presentation were called for inclusion in this group. This group was studied to determine the relationship of *H. pylori* to recurrent duodenal ulceration in the long term following simple closure of perforated duodenal ulcer. A total of sixty patients formed this group. The patients were randomly selected from previous operation records. In these patients, history and clinical examination was noted according to the proforma (Annexure-IV). An upper gastrointestinal endoscopy was done at the first presentation and the findings were noted. The *H. pylori* status was determined by urease test, histology and serology.

The prevalence rate of *H. pylori* in these patients was compared between those with or without presence of an ulcer on endoscopy. The prevalence rate of *H. pylori* was also related to gender, size of ulcer, smoking and alcohol abuse. The scheme of study in this group is shown on the next page.
GROUP IVB – DUODENAL ULCER PERFORATION
(RETROSPECTIVE CASES)

DUODENAL ULCER PERFORATION

SIMPLE CLOSURE -> 5 YEARS EARLIER

ENDOSCOPY, UREASE TEST, SEROLOGY, HISTOLOGY AT PRESENTATION

PREVALENCE OF H. PYLORI

CORRELATION WITH RECURRENCE OF ULCER
METHODS:

Endoscopy:

After overnight fasting and informed consent, all patients were subjected to upper gastrointestinal endoscopy using a Pentax Video endoscope (EG 3400) under topical anaesthesia using 2% xylocaine jelly in adults and dissociated general anaesthesia with intravenous ketamine in children under 12 years of age. Defoaming agents were not used as these are known to suppress H pylori. Using a standard endoscopic biopsy forceps, two gastric mucosal biopsies were taken from within 2 cms of the pylorus. Four biopsy bits were taken, two each for the urease test and histology for detection of H pylori. The endoscope and biopsy forceps were sterilized between patients using 2% glutaraldehyde solution as per recommendations (Cidex-Johnson and Johnson). Following sterilization, the endoscope and biopsy forceps were cleaned with sterile saline as residual glutaraldehyde may also produce suppression of H pylori.

Urease test:

This was done by a urea solution prepared and standardized in our institution [267]. It contained 250 mg urea, 400 μl of gentamicin (40 mg/ml) and 400 μl of phenol red in 15 ml of distilled water. 1 ml of this solution was then placed in a 5 ml screw capped bottle.
The sensitivity and specificity of this urea solution was 93% and 92% respectively compared to histology. The change of colour from yellow to pink read upto 24 hours at room temperature (37°C) after inoculating two endoscopic gastric mucosal biopsies from within 2 cm of pylorus was considered positive. Though there is a possibility of uneven or patchy distribution of *H pylori* throughout the gastric mucosa, the gastric antrum appears to be more uniformly involved and two biopsies taken from within 2-5 cm of pylorus are generally held to be sufficient for diagnosis [74,268].

Serology:

The serum assay used for the detection of IgG antibodies to *H pylori* was a validated second generation Microwell ELISA assay using purified solid phase antigens (UBI Magiwel *Helicobacter pylori* IgG Quantitative IA-601 Microwell ELISA supplied by United Biotech Inc., CA, USA).

5 ml of blood was collected from each patient by standard venipuncture, labelled and sera was separated and was stored at -20°C till the test was done. The labelling was done with a coded number so that the procedure was blinded during the ELISA technique. The numbers were only decoded while analysing the results. The test was considered valid if it met the given criteria. This commercial kit is mentioned to have a sensitivity and specificity of 98% and 84% respectively compared to culture. The sensitivity and specificity compared to the CLO urease test was 96% and 84% respectively. When compared to histology the sensitivity and
specificity was 97% and 93% respectively (Information supplied by the manufacturer). This test was validated at our centre and found to have a sensitivity of 91% and a specificity of 84% compared to the urease test. On comparison with histology the sensitivity and specificity was 93% and 88% respectively.

Principle of the assay:

The Magiwel H pylori IgG is a microwell ELISA for the detection of IgG antibodies to H pylori in human serum. The wells coated with purified H pylori antigens were used. Diluted patient serum and reference controls are incubated in the wells. The H pylori IgG specific antibody, if present, binds to the solid phase antigens. All unbound antibody is washed off and the enzyme conjugated anti-human IgG is added. The enzyme conjugate is then bound to the antigen-antibody complex. The unbound enzyme conjugate is washed off. Upon addition of substrate and chromogen, the intensity of colour developed is proportional to the concentration of anti- H pylori IgG in the samples.

Assay procedure:

The entire kit was used during each session of ELISA testing for serology. 88 samples were tested in each session. The coated wells were secured in the holder with sample identification. 1:101 dilutions of test samples, negative, positive and calibrator were prepared by adding 5 μL of sample to 0.5 ml of sample of diluent in separate glass tubes. 100 μL of diluted control patient samples and sample diluent (as the blank) were dispensed into each coated well. After incubation for 30 minutes
at room temperature, the solution was discarded and the wells rinsed 7 times with washing buffer. Thereafter 100 µL of enzyme conjugate (anti-human IgG conjugated with horse radish peroxidase) was dispensed to each well and incubated for 30 minutes at room temperature. The solution was discarded and the wells rinsed 7 times with washing buffer. 100 µL of buffer solution containing hydrogen peroxide and 100 µL of tetramethyl benzidine were dispensed into each well. After incubation for 15 minutes at room temperature, the reaction was stopped by adding 50 µL of 1 N sulphuric acid into each well. The optical density (OD) was read at 450 nm with a microwell reader.

Calculation of results:

The Microwell reader which was capable of determining absorbance at 450 nm was used. The IgG value of anti- H pylori in each patient was obtained as follows:

\[
\text{IgG titre in Elisa Units (EU)} = \frac{(\text{O.D. of test sample}) \times (\text{EU/ml of calibrator})}{\text{per ml of sample} \times \text{OD of calibrator}}
\]

Quality control:

The test run was considered valid provided the following criteria were met:

1. Optical density value of reagent blank was less than 0.2
2. Optical density value of calibrator was greater than or equal to 0.6
3. The EU/ml values of negative and positive controls were within the range printed on the labels (negative control – less than 30 EU/ml; positive control – 40-90 EU/ml).
Whenever the quality control did not meet the above criteria, the test was not valid and the whole procedure was repeated.

Interpretation of ELISA test:

1. Negative: Less than 30 EU/ml. Absence of detectable levels of \textit{H pylori} antibodies.

2. Equivocal: Between 30-40 EU/ml. This required retest with another kit. In all equivocal samples repeat tests were done till a positive or negative interpretation was achieved.

3. Positive: Higher than 40 EU/ml. Positive for the presence of IgG specific to \textit{H pylori}.

Histology:

Two endoscopic gastric mucosal biopsies taken from pyloric antrum within 2 cm of pylorus were fixed in 10% buffered formation. Two corpus biopsies were taken in Group IVA patients to assess \textit{H pylori} state following treatment. Paraffin embedded histological sections were stained with giemsa stain for identification of \textit{H pylori} [269]. Two pathologists blinded to the clinical history, endoscopic findings, urease and serology results examined the tissue sections for the presence of \textit{H pylori} organisms. The \textit{H pylori} status by Giemsa stain was considered positive if it was reported positive by one or both pathologists. The \textit{H pylori} state was negative if both the pathologists reported the slide as negative for \textit{H pylori}.
Method for Giemsa staining:

Solutions:

a) GIEMSA STOCK SOLUTION

Giemsa stain powder  4 g
Glycerol  250 ml
Methanol (pure)  250 ml

Dissolve the powder in the glycerol at 60°C with regular shaking. Add methanol, shake the mixture and allow to stand for 7 days. Filter before use.

b) WORKING GIEMSA STAIN

Giemsa stock solution  4 ml
Acetate buffer distilled water, pH 6.8  96 ml

Method:

1. Bring sections down to water through graded alcohol
2. Rinse in distilled water
3. Stain in working Giemsa stain, overnight
4. Rinse in distilled water
5. Rinse in 0.5% aqueous acetic acid until section is pink
6. Wash in distilled water
7. Dehydrate rapidly through alcohols, clear in xylene and mount in DPX (Distrene polyol xylene)
Results:

Micro-organisms - dark blue
Background - pink to pale blue
Nuclei - blue

ASSESSMENT OF *H PYLORI* STATUS:

The *H pylori* status was determined by urease test, serology for IgG antibodies against *H pylori* and histology using Giemsa stain [270]. Serology was used as one of the methods of determining *H pylori* status as *H pylori* is a chronic infection that does not resolve spontaneously and elevated titres indicate current infection, unless the patient is known to have been treated for *H pylori* in the recent past [38]. *H pylori* specific serum IgG response is both highly specific (99%) and sensitive (96%) in detecting *H pylori* colonization [116]. Culture was not done as the sensitivity is moderate even though it is specific [75]. The *H pylori* status was determined in the various groups as follows.

1.(a)Group IA:

*H pylori* status was determined by serology. As general anaesthesia would be required in most of these patients for doing endoscopy, it was considered unethical to subject them for endoscopy and invasive tests for detection of *H pylori*. 
(b) Group IB:

Any two of the above mentioned three tests, if positive was taken as a positive *H pylori* state [268,271-272]. If only one test was positive then it was considered as negative for *H pylori* status.

2. Group II and Group III:

Any two of the above mentioned three tests, if positive was taken as a positive *H pylori* state.

3. (a) Group IVA:

Within three days of admission for perforated duodenal ulcer, 5 ml of venous blood was taken for *H pylori* serology. At six week follow up (about 8 weeks from presentation) a further blood sample was obtained from these patients for repeat *H pylori* serology. The possibility that acute perforated duodenal ulceration could be associated with the early phase of *H pylori* infection before IgG seroconversion has occurred must be considered; hence repeat serology at 8 weeks was necessary as some patients may be negative serologically at presentation when infection is recently acquired and become positive by 8 weeks. Reinbach et al had used a similar procedure for documenting *H pylori* in patients with perforated duodenal ulcer [25]. Per operative endoscopy, urease test and histology were not done in these patients due to ethical problems. Antral biopsies through the duodenal perforation for urease test and histology were also not done for ethical reasons as there was a
possibility of increasing the size of perforation. During follow up, urease and histology was done to determine eradication of *H pylori*. A negative urease and histology test was defined as an eradicated status.

(b) Group IVB:

Urease, histology and serology was carried out in this group. Any two of the three tests if positive was considered as indication of a positive *H pylori* status.

**STATISTICAL ANALYSIS:**

Analysis of data was done using Epi Info Software (Version 6.04) [273]. The logistic regression analysis was done using SPSS windows (Version 6.0)

(1) Chi-square ($X^2$) for linear trend was used to evaluate age related prevalence of *H pylori* in asymptomatic controls and patients with perforated peptic ulcer.

(2) Chi-square test and Fisher's exact test (where appropriate) were used to compare the prevalence of *H pylori* in various upper GI disorders with the prevalence seen in asymptomatic adults and children. This test was also used to relate the prevalence rate of *H pylori* to various parameters like NSAID use, smoking, alcohol abuse, number, size and type of ulcers. It was also used to compare the prevalence of *H pylori* in uncomplicated and complicated duodenal ulcers. When a significant difference was noted, a further analysis was done with adjustment for age and gender using logistic regression analysis.
Chi-square test and Fisher's exact test were used to correlate the prevalence of *H. pylori* in perforated duodenal ulcer to parameters such as gender, size, chronicity, smoking and alcohol abuse. These tests were used to determine and correlate fall in serological titres and eradication of *H. pylori*. The tests were also used for comparison of the effect of ranitidine and quadruple therapy on eradication of *H. pylori* and for correlation of eradication with ulcer recurrence. When a significant difference was noted, further analysis was done with adjustment for age and gender using logistic regression analysis.

Comparison of anti-*H. pylori* IgG titres were made using one way analysis of variance (ANOVA) and Student's 't' test where appropriate.

For all these tests, a calculated alpha value of less than 5% was considered significant.

**ETHICAL CLEARANCE:**

The study was approved by the Research Council and the Ethics Committee of the Institute where the study was conducted.

The overall scheme of methodology in this study is shown on the next page.
## OVERALL SCHEME OF METHODOLOGY RELATED TO AIMS

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<td>Urease test, anti-<em>H. pylori</em> IgG serology and histology (Giemsa stain) Any 2 out of 3, if positive, indicates positive <em>H. pylori</em> status in adults In children only serological titres of IgG to establish positive <em>H. pylori</em> status</td>
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<td>2. Determine normal prevalence</td>
<td>Establish <em>H. pylori</em> prevalence in a) Children (n=105): History and examination only to exclude upper alimentary disorders b) Adults (n=100): Upper gastrointestinal endoscopy (UGIE) to exclude upper alimentary disorders</td>
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<tr>
<td>3. Establish prevalence in upper gastrointestinal disorders</td>
<td>Inclusion of patients with following upper gastrointestinal disorders documented and proved by UGIE + histology whenever required A = Duodenal ulcer (n = 166) B = Gastric ulcer (n = 53) C = Non-ulcer dyspepsia (n = 50) D = Carcinoma stomach (n = 51) E = Chronic gastritis (n = 50) F = Erosive gastroduodenitis (n = 58) G = Stomal ulcer (n = 30) Prevalence in these disorders compared to asymptomatic controls</td>
</tr>
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<td>4. Determine prevalence in complications of duodenal ulcer other than perforation</td>
<td>Inclusion of patients with complications of duodenal ulcer A = Bleeding duodenal ulcer (n = 30) B = Duodenal ulcer with gastric outlet obstruction (n=112) Prevalence compared to uncomplicated duodenal ulcer and controls</td>
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<tr>
<td>5. Assess relationship to duodenal ulcer perforation</td>
<td>Short and medium term recurrence (upto 2 years) Inclusion of patients with duodenal ulcer perforations who underwent simple closure on prospective basis (n = 202) Initial prevalence established by anti-<em>H. pylori</em> IgG serology Patients randomized to receive Ranitidine / Quadruple therapy <em>H. pylori</em> eradication rates studied by UGIE, urease and histology 6 monthly followup to maximum of 2 years Correlation of <em>H. pylori</em> positivity rate to ulcer recurrence</td>
</tr>
<tr>
<td>6. Value of fall in serological titres in establishing eradication</td>
<td>Long term recurrence (over 5 years) Inclusion of patients with duodenal ulcer perforation who underwent simple closure more than 5 years prior to presentation (n = 60) Prevalence established by urease test, histology and serology Prevalence of <em>H. pylori</em> correlated with recurrence of ulcer</td>
</tr>
<tr>
<td></td>
<td>Study of sensitivity, specificity, positive predictive value, negative predictive value and accuracy of declining serological titres compared to baseline using cutoff values at 10%, 20%, 25%, 30% and 50%</td>
</tr>
</tbody>
</table>
Positive urease test

Negative urease test
ELISA for serology: H. pylori antigens coated 96 wells on Microwell strips with enzyme conjugate

ELISA: test in progress
ELISA: Golden yellow colour showing a positive test for H.pylori

ELISA: Microwell reader
Histology: Giemsa stain for H. pylori (X 1000)

H. pylori in its natural habitat under the gastric mucus layer (Giemsa X 1000)