MATERIALS AND METHODS
SELECTION OF PATIENTS:

The study was undertaken at SHREE P. M. PATEL COLLEGE OF PARAMEDICAL SCIENCE AND TECHNOLOGY, ANAND from February 2008 to August 2011.

Approval of Human Ethical Review Committee of Pramukh Swami Medical College (HREC), Karamsad, was taken prior to initiation of the work. Dully filled consent form was obtained by all the patients participating in the study.

Total 855 symptomatic cases of gastro intestinal disorders were processed for detection of H.pylori infection. Most of the patients were suffering from Gastritis, Duodenitis, Duodenal ulcer, Reflux esophagitis, etc. with complain of abdominal pain, nausea, vomiting, persistence gnawing, hematemesis, etc.

Exclusion criteria were the following: 1) informed consent was not obtained 2) There was a recent or active gastrointestinal bleeding 3) Previous therapy to eradicate Helicobacter pylori 4) Patients taking aspirin or non-steroidal anti-inflammatory drugs (NSAIDS) in the past 4 weeks or are on PPI. 5) Previous surgical procedure on digestive tract. 6) Other severe accompanying diseases.

SAMPLE COLLECTION:-

Biopsy Samples for processing were collected at DEEP SURGICAL HOSPITAL, ANAND.

BIOPSY:

Total four antral biopsies were collected from each symptomatic patient in fasting condition after giving local anesthesia spray in
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Pharyngeal region. Upper gastrointestinal endoscopy was done using OLYMPUS VIDEO ENDOSCOPE 1306. After collection, all the four antral biopsies were collected in sterile Brain Heart Infusion broth for safe transfer to processing laboratory.

BLOOD SAMPLE:

2-3 ml of blood is collected for serological test. Blood is collected either in plain bulb or in EDTA bulb.

TRANSPORT OF SPECIMEN

Biopsy specimen is transported immediately to the processing laboratory without delay. Storage before processing: If delay in processing is unavoidable the biopsy specimen can be store in refrigerator for an hour at -4°C before culture. Urea broth is stored at room temperature.

PROCESSING OF SAMPLE:

On arrival of sample, first of all it is to be labeled with patient’s identification no., date, time of collection, all information about patient which is to be entered in record.

1) RAPID UREASE TEST:

- Reagent preparation UREA BROTH:
  - Peptone ..........................................................1g
  - Sodium chloride .............................................5g
  - Dipotassium hydrogen phosphate.........................2g
  - Phenol red (1:500 aqueous solution) .......................6ml
  - Distilled water ...............................................1L
  - Urea, 20% solution, sterile ..................................100ml
All the above ingredients except glucose and urea were mixed in a bottle; pH was adjusted to 6.8 to 6.9 and sterilized by autoclaving at 121°C.

For 30 min, it was cooled to about 50°C and then sterile solution of glucose and urea were added. The broth was then dispensed in 0.5ml quantity in sterile vials, and refrigerated at 4°C until used.

Principle of test: - Urease producing organism decomposed urea to ammonia, which in turn produces alkaline condition of medium indicated by color change of phenol red from yellow to pink color.

\[
\text{NH}_2 \text{CO NH}_2 + \text{H}_2\text{O} \rightarrow 2\text{NH}_3 + \text{CO}_2
\]

Method: - After insertion of gastric biopsy, vial was kept at room temperature up to 24 hours and time taken for color changes from yellow to pink was noted.

2) CULTURE

Reagent preparation of CULTURE MEDIA

A. Brain Heart Infusion broth, Hi Veg. : (HIMEDIA) MV-210-500G

- Ingredients
  
  Grams/liter
  
  - Hi Veg. Special infusion ......................... 0.750
  - Hi Veg. Infusion ................................. 10.00
  - Hi Veg. Peptone No.3 ......................... 10.00
  - Dextrose ................................. 0.200
  - Sodium chloride ......................... 0.500
  - Disodium phosphate .................. 0.250

  Final pH (at 25°C) 7.4 ± 0.2
Suspend 37.0 grams in 1000ml D/W. Heat if necessary to dissolve the medium completely. Dispense in to screw capped bottles or as desired. Sterilized by autoclaving at 15 lbs. pressure (121°C) for 15 minutes.

B) Brucella blood agar: Brucella Hi Veg Agar Base (MV 074-500G)

- **Ingredients**
  - Grams/liter
  - Hi Veg hydro lysate……………………………10.00
  - Hi Veg peptone…………………………………10.00
  - Yeast Extract…………………………………02.00
  - Dextrose……………………………………01.00
  - Sodium chloride……………………………….05.00
  - Sodium bisulphide……………………………0.10
  - Agar………………………………………15.00

Final pH (at 25°C) 7.0 ± 0.2

Add extra 1.5 g agar powder in each 100ml of media. Suspend 21.55g of media in 500 ml distilled water. Heat to boil, so as to dissolve the medium completely. Sterilize by autoclaving at 15lbs pressure (121°C) for 15 minutes Cool to 45°C – 50°C before adding heat sensitive compounds.

Add 5% v/v sterile defibrinated sheep blood or human blood to sterile cooled base. Add 2 ml of campylobacter supplement- Ш (Skirrow) in 500 ml of melted media & mix properly & pour in sterile Petri dishes.

C) Belo Horizonte agar:-

Composition: - (Brain Heart infusion agar + 5 -10% defibrinated blood of human or sheep. + 2, 3, 5-Triphenyl tetrazolium chloride + Campylobacter supplement-Ш (Skirrow))
MATERIALS AND METHODS

Campylobacter Supplement (Skirrows III):-

An antibiotic supplement recommended for selective isolation of Campylobacter species.

Formula: (per vial is sufficient for 500ml medium)

- Polymyxin B 1,250 IU
- Vancomycin 5.00 mg
- Trimethoprim 2.50 mg

Directions: - Rehydrate the content of one vial aseptically with 2ml of sterile D/W and mix well to dissolve. Avoid frothing of the solution. Aseptically & the contents to 500ml of sterile, molten Blood Agar Base with 5-7% v/v defibrinate human or sheep blood to prepare Brucella blood agar, Belo Horizonte agar. Also add in molten sterile modified chocolate agar.

METHOD:-

Biopsy specimen is rubbed in form of well on different solid media used and then streaked by four flame method. Then the media are incubated in anaerobic jar with providing gas pack kit for *H.pylori* which provides suitable atmospheric condition necessary for growth of *H.pylori*. The jar is then incubated at 37°C for at least 5-7 days. Growth of *H.pylori* is identified by gram negative appearance, positive oxidase and catalase test and shows rapid urease test positive.

- Various media are used for culture of *H.pylori* :-
  - Brucella blood agar
  - Modified chocolate agar
  - Belo Horizonte medium
Identification on culture medium (After incubation of 5-7 days):

- **Brucella blood agar** → minute, translucent, round, convex colonies with entire edges.
- **Modified chocolate agar** → small, round, opaque, golden colonies with entire edges.
- **Belo Horizonte medium** → tiny, round, opaque, golden colonies with entire edges.

All the colonies are confirmed as of *H. pylori* by gram negative appearance, positive oxidase and catalase tests & a rapidly positive urease test.

3) **GRAM’S STAINING** :

**PRINCIPLE:** The gram positive organism after staining with primary basic dye and treatment with mordent resist decolorization by Gram’s decolorizer and appear violet in color, while Gram negative organism decolorized by decolorizer and are stain with counter stain and appear pink in color.

**Preparation of smear:** Smear is prepared on clean glass slide by rubbing the biopsy sample. The smear is allowed to dry and then heat fixed.

- **Staining of smear:**
  - Cover the smear with Gentian Violet and allow standing for 1 minute.
  - Pour off the Gentian violet and cover the smear with Gram’s iodine for 1 minute.
  - Wash the slide with water.
MATERIALS AND METHODS

- Decolorize the smear with the help of Gram’s decolorizer until the violet color come out from the smear.
- Wash the slide with water.
- Cover the smear with the counter stain like saffranin and allow it to stand for 5 minutes.
- Wash the slide with water and allow the smear to air dry.
- Examine the smear in oil immersion.
- **Result:** *H. pylori* appear as helical or spiral shaped Gram negative rods.

4) SEROLOGICAL TEST:

- **ImmunoComb® II: Helicobacter pylori IgG**
  
  ORGENICS CE  Code: 60425002  Format 3 x 12 Test

  **Principle:** The ImmunoComb II *Helicobacter pylori* IgG test is an indirect solid–phase Enzyme immunoassay (EIA)

- The solid phase is card with 12 projections (teeth). Each tooth is seen sensitized at two positions.
- The developing plate has six rows (A–F) of twelve wells, each row containing a reagent solution ready for use at different step in the assay. The test is performed stepwise moving the card from row to row, with incubation at each step.
- At the outsets of the test serum or plasma specimen are pre diluted 1:11 and added to the diluents in the well of row A of the developing plate.
- The card is then inserted into the wells of the row A. Antibodies to *H. pylori*, is present in the specimen will specifically bind to the *H. pylori* antigen on the lower spot on the teeth of the card.
- Simultaneously, immunoglobulin presents in the specimen will be captured by the antihuman immunoglobulin on the upper
spot (internal control). Unbound components are washed away in row B. In row C the anti *H. pylori* IgG captured on the teeth will react with antihuman IgG labeled with Alkaline phosphatase in the next two rows, unbound components are removed by washing. In row F, the bound alkaline phosphatase reacts with chromogenic components. The results are visible as gray blue spot on surface of teeth of the card.

**Immunocomb showing different titers from 20 to 160 IU/ml**

* Upper spot: goat antibodies to Human immunologic (internal control).

* Lower spot: antigens of inactivated *H. pylori*. 
Developing Plate:

The kit contains three developing plates covered with aluminum foil. Each developing plate contains all reagents needed for test. The developing plate consists of six rows (A-F) of 12 wells each.

The contents of each row are as follows:

- Row A: Specimen diluent
- Row B: Washing solution
- Row C: Goat antihuman IgG antibodies labeled with alkaline phosphatase
- Row D: Washing Solution
- Row E: Washing Solution
- Row F: Chromogenic Substrate Solution containing 5-bromo 4-chloro 3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT).

Storage and Stability of the Kit:

Performance of the kit after the first opening is stable up to the expiry date of the kit, when stored at 2-8°C.

TEST PROCEDURE:

Preparing the developing plate:

1. Incubate the developing plate in an incubator at 37°C for 20min or leave at room temperature (22°C-26°C) for 3hrs
2. Cover the work table with absorbent tissue to be discarded as biohazard test at the end of the test.
3. Mix the reagents by gently shaking the developing plate
MATERIALS AND METHODS

Immunocomb kit with all reagents

Procedure:

1) Tear the aluminum pouch of the card at the notched edge.

2) Remove the card. Cut the required number of card.

Predilution of specimens and controls:

3) For each specimen and control, dispense 100 μl of specimen diluent in to a microtube or microtitre well.

4) To each micro tube or well, add either 10 μl of a specimen, or 10μl of the positive control or negative control supplied with the kit. Mix by repeatedly refilling and ejecting the solution.
**Materials and Methods**

**Antigen-Antibody reaction:**

5) Pipette 25 μl of a prediluted specimen. Perforate the foil cover of one well of row A of the developing plate with the pipette tip or perforate and dispense the specimen at the bottom of the well. Mix by repeatedly refilling and ejecting the solution.

6) Repeat step 5 for the other prediluted specimens and the two prediluted controls.

7) Use a new well in row A for each specimen or control. Change Pipette tips between specimens.

A. Insert the card in to the well of row A containing specimens and controls.

**Mix:** Withdraw and insert the card in the wells several times.

B. Leave the card in row A for exactly 30 min.

C. At the end of 30 minutes take the card out of row A. Absorb adhering liquid from the pointed tips of the teeth on clean absorbent paper. Do not touch the front surface of the teeth.

**First wash (Row B)**

8) Insert the card in to the well of Row B. Agitate: vigorously withdraw and insert the card in the well for at least 10 sec. to achieve proper washing. Repeat agitation several times during the course of 2 minutes; meanwhile perforate the foil of Row C.

After 2 minutes withdraw the card and absorb adhering liquid.
MATERIALS AND METHODS

Binding of conjugate: (Row C)

9) Insert the card into the wells of row C. Mix, After 20 min withdraw the card and absorbed adhering liquid.

Second wash (Row D)

10) Insert the card into the wells of row D. repeatedly agitate during 2 Minute as in step 6. Meanwhile perforate the foil of row E. After 2 min, withdraw the card and absorb adhering liquid.

Third wash (Row E)

11) Insert the card in to the wells of row E. Repeatedly agitates during 2 Minutes, Meanwhile perforate foil of row F. After 2 min withdraw the Card and absorb adhering liquid.

Color reaction (row F)

12) Insert the card in the wells of row F. Mix. Set the timer for 10 minutes. After 10 minutes, withdraw the card.

Stop reaction (row E)

13) Insert the card again in to row E. After one minute withdraw the card and allow it to dry in the air.

14) Note down the titer.
ELISA (IgG / IgA)

PRINCIPLE OF THE ASSAY: The quantitative immunoenzymatic determination of IgG/IgA-class antibodies against Helicobacter pylori is based on the ELISA (Enzyme-linked Immunosorbent Assay) technique.

Microtiter strip wells are precoated with Helicobacter pylori antigens to bind corresponding antibodies of the specimen. After washing the wells to remove all unbound sample material horseradish peroxidase (HRP) labeled anti-human IgG/IgA conjugate is added. This conjugate binds to the captured Helicobacter specific antibodies. The immune complex formed by the bound conjugate is visualized by adding Tetramethylbenzidine (TMB) substrate which gives a blue reaction product.

The intensity of this product is proportional to the amount of Helicobacter specific IgG antibodies in the specimen. Sulphuric acid is added to stop the reaction. This produces a yellow endpoint colour. Absorbance at 450 nm is read using an ELISA microwell plate reader.

1) MATERIALS

   - Reagents supplied

     - **Helicobacter pylori Coated Wells (IgG/IgA):** 12 break apart 8-well snap-off strips coated with Helicobacter pylori antigen; Vacuum sealed, in resealable aluminum foil.

     - **IgG/IgA Sample Diluent ***:** 1 bottle containing 100 ml of buffer for sample dilution; pH 7.2 ± 0.2, colored yellow; ready to use; White cap.

     - **Stop Solution:** 1 bottle containing 15 ml sulphuric acid, 0.2 mol/l; ready to use; red cap.
**MATERIALS AND METHODS**

- **Washing Solution (20x conc.)***: 1 bottle containing 50 ml of a 20-fold concentrated buffer for washing the wells; pH 7.2 ± 0.2, White cap.
- **Helicobacter pylori anti-IgG/IgA conjugate**: 1 bottle containing 20 ml of peroxidase labeled antibodies to human IgG/IgA. Colored red; ready to use; black cap.
- **TMB Substrate Solution**: 1 bottle containing 15 ml 3, 3', 5, 5'-tetra-methyl-benzidine (TMB); ready to use; yellow cap.
- **Helicobacter pylori IgG/IgA Standards**: 4 vials, each containing 2 ml; ready to use:
  - Standard A: 0 NTU/ml; blue cap
  - Standard B: 15 NTU/ml; green cap
  - Standard C: 75 NTU/ml; yellow cap
  - Standard D: 150 NTU/ml; red cap

  * contains 0.01 % Kathon after dilution, ** contains 0.2 % Bronidox L, *** contains 0.1 % Kathon

- **Materials supplied**
  - 1 Strip holder
  - 2 Cover foils
  - 1 Test protocol
  - 1 distribution and identification plan.

- **Materials and Equipment needed**
  - ELISA micro well plate reader, equipped for the measurement of absorbance at 450/620nm
MATERIALS AND METHODS

- Incubator 37°C
- Manual or automatic equipment for rinsing wells
- Pipettes to deliver volumes between 10 and 1000 μl
- Vortex tube mixer
- Deionized or (freshly) distilled water
- Disposable tubes Timer

2) STABILITY AND STORAGE

The reagents are stable up to the expiry date stated on the label when stored at 2...8 °C.

3) REAGENT PREPARATION

It is very important to bring all reagents, samples and standards to room temperature (20…25°C) before starting the test

3.1. Coated snap-off Strips

The ready to use break apart snap-off strips are coated with Helicobacter pylori antigen. Store at 2-8°C. The strips are vacuum sealed. Immediately after removal of strips, the remaining strips should be resealed in the aluminum foil along with the desiccant supplied and stored at 2...8 °C; stability until expiry date.

3.2. Helicobacter pylori anti-IgG Conjugate

The bottle contains 20 ml of a solution with anti-human IgG/IgA horseradish peroxidase, buffer, stabilizers, preservatives and an inert
red dye. The solution is ready to use. Store at 2…8°C. After first opening until expiry date when stored at 2…8°C.

3.3. Standards

The vials labeled with Standard A, B, C and D contains a ready to use standard solution.

The concentrations of the standards are:

- **Standard A:** 0 NTU/ml
- **Standard B:** 15 NTU/ml
- **Standard C:** 75 NTU/ml
- **Standard D:** 150 NTU/ml

The solutions have to be stored at 2…8°C and contain 0.1% Kathon. After first opening until expiry date when stored at 2…8°C.

3.4. IgG/IgA Sample Diluent

The bottle contains 100 ml phosphate buffer, stabilizers, preservatives and an inert yellow dye. It is used for the dilution of the patient specimen. This ready to use solution has to be stored at 2…8°C. After first opening until expiry date when stored at 2…8°C.

3.5. Washing Solution (20xconc.)

The bottle contains 50 ml of a concentrated buffer, detergents and preservatives. Dilute washing solution 1+19; e.g. 10 ml washing solution + 190 ml fresh and germ free redistilled water. The diluted buffer will keep for at least four weeks if stored at 2…8°C.

*Crystals in the solution disappear by warming up to 37 °C in a water bath.*
MATERIALS AND METHODS

3.6. TMB Substrate Solution

The bottle contains 15 ml of a tetramethylbenzidine/hydrogen peroxide system. The reagent is ready to use and has to be stored at 2…8°C, away from the light. The solution should be colorless or could have a slight blue tinge. If the substrate turns into blue, it may have become contaminated and should be thrown away. After first opening until expiry date when stored at 2…8°C.

3.7. Stop Solution

The bottle contains 15 ml 0.2 M sulphuric acid solution (R 36/38, S 26). This ready to use solution has to be stored at 2 … 8°C.

4) SPECIMEN COLLECTION AND PREPARATION

Use human serum samples with this assay. If the assay is performed within 24 hours after sample collection, the specimen should be kept at 2…8°C; otherwise they should be aliquot and stored deep-frozen (-20 to -70°C). If samples are stored frozen, mix thawed samples well before testing.

Avoid repeated freezing and thawing.

4.1. Sample Dilution

Before assaying, all samples should be diluted 1+100 with IgG Sample Diluent. Dispense 10μl sample and 1ml IgG Sample Diluent into tubes to obtain a 1+100 dilution and thoroughly mix with a Vortex. Standards are ready to use and must not be diluted.
5) ASSAY PROCEDURE

5.1. Test Preparation

Please read the test protocol carefully before performing the assay. Result reliability depends on strict adherence to the test protocol as described. Prior to commencing the assay, the distribution and identification plan for all specimens and standards should be carefully established on the result sheet supplied in the kit. Select the required number of microtiter strips or wells and insert them into the holder. Please allocate at least:

1 well (e.g. A1) for the substrate blank,

4 wells (e.g. B1, C1, etc.) for Standard A, B, C and D.

It is recommended to determine standards and patient samples in duplicate.

Perform all assay steps in the order given and without any appreciable delays between the steps.

A clean, disposable tip should be used for dispensing each control and sample.

Adjust the incubator to 37°C ± 1°C.

1. Dispense 100μl of each Standard (A, B, C and D) and diluted samples into the respective wells. Leave well A1 for substrate blank.

2. Cover wells with the foil supplied in the kit.

3. Incubate for 1 hour ± 5 min at 37±1°C.

4. When incubation has been completed, remove the foil, aspirate the content off the wells and wash each well three times with 300μl of washing solution. Avoid overflows from the reaction wells. The soak

H.pylori
MATERIALS AND METHODS

time between each wash cycle should be >5sec. At the end carefully remove remaining fluid by tapping strips on tissue paper prior to the next step!

Note: Washing is critical! Insufficient washing results in poor precision and falsely elevated absorbance values.

5. Dispense 100μl Helicobacter pylori anti-IgG Conjugate into all wells except for the blank well (e.g. A1). Cover with foil.

6. Incubate for 30 min at room temperature (20 to 25°C). Do not expose to direct sunlight.

7. Repeat step 4.

8. Dispense 100μl TMB Substrate Solution into all wells

9. Incubate for exactly 15 min at room temperature (20 to 25°C) in the dark.

10. Dispense 100μl Stop Solution into all wells in the same order and at the same rate as for the TMB Substrate Solution.

Any blue color developed during the incubation turns into yellow.

Note: Highly positive patient samples can cause dark precipitates of the chromogen! These precipitates have an influence when reading the optical density. Predilution of the sample with physiological sodium chloride solution, for example 1+1, is recommended. Then dilute the sample1+100 with dilution buffer and multiply the results in NTU by 2.

11. Measure the absorbance of the specimen at 450/620nm within 30 min after addition of the Stop Solution.
5.2. Measurement

Adjust the ELISA Micro well Plate Reader to zero using the substrate blank in well A1.

If - due to technical reasons - the ELISA reader cannot be adjusted to zero using the substrate blank in well A1, subtract the absorbance value of well A1 from all other absorbance values measured in order to obtain reliable results!

Measure the absorbance of all wells at 450 nm and record the absorbance values for each control and patient sample in the distribution and identification plan.

Dual wavelength reading using 620 nm as reference wavelength is recommended.

Where applicable calculate the mean absorbance values of all duplicates.

6) RESULTS

6.1. Assay validation criteria

In order for an assay to be considered valid, the following criteria must be met:

Substrate blank in A1: Absorbance values lower than 0.100.

Standard A in B1: Absorbance values lower than 0.2.

6.2. Calculation of Results

In order to obtain quantitative results in NTU/ml blot the (mean) absorbance values of the 4 Standards A, B, C and D on (linear/linear) graph paper in a system of coordinates against their corresponding concentrations (0, 15, 75 and 150 NTU/ml) and draw a standard
calibration curve (absorbance values on the vertical y-axis, concentrations on the horizontal x-axis).

Read results from this standard curve employing the (mean) absorbance values of each patient specimen and control.

All suitable computer programs available can be used for automated result reading and calculation.

### 6.3. Interpretation of Results

Normal value ranges for this ELISA should be established by each laboratory based on its own patient populations in the geographical areas serviced.

- The following values should be considered as a guideline:
  - **Reactive**: > 20 NTU/ml
  - **Grey zone (equivocal)**: 15 - 20 NTU/ml
  - **Non-reactive**: < 15 NTU/ml
5) HISTOPATHOLOGICAL TECHNIQUES:

- **Tissue Processing for Histopathological Examination**

1. **Fixation**

Specimen bits are placed in porous cassettes it should not more than 5mm thick. Ideal fixative used is 10% formal saline.

2. **Dehydration**

After fixation, place cassettes into acetone in gradual sequence (70, 95, and 100%) to make way for paraffin for a three hour.

3. **Clearing**

Removal of acetone with “xylene” that will be miscible with the embedding medium (paraffin) and then Impregnating with paraffin. It Makes tissue appear “clear” (1 hr+1hr)

4. **Embedding**

Replace xylene with paraffin .for that put tissue in melted paraffin at 55° C (M.P of wax). Remove all bubbles and give two baths of melted paraffin for 1 hour each.

5. **Block making**

After two hour of wax embedding, with the help of tissue tack make a block to cut, this Paraffin blocks than taken for sectioning.
6. Sectioning

For sectioning trimmed block for excess paraffin removed and block face in a trapezoid shape. Place tissue block in microtome with wide edge of trapezoid lowest, and parallel to knife and Advance blade toward block. Cut the section at 5-8 micron size.

7. Staining

Stain the slide for different histological staining technique like H & E, Giemsa and Warthin-starry

8. Mounting

Mount the slide with the help of mounting media DPX.

Staining Technique

(1) HAEMATOXYLIN & EOSIN STAIN (H&E):

Haematoxylin is a charge-based, general purpose stain. Haematoxylin stains acidic molecules shades of blue. Eosin stains basic materials shades of red, pink and orange. H & E stains are universally used for routine histological examination of tissue sections.

* Haematoxylin: Extracted from logwood of the tree Haematoxylon campechianum.

Haematoxylin

Haematein (dye) + Alum (Mordant) + Base (Tissue)
MATERIALS AND METHODS

Progressive attachment of mordant to dye and tissue to dye mordant. Glycerol has been incorporated into many formulae for its value in preventing over oxidation and reducing evaporation.

PROTOCOL

- Remove paraffin wax with xylene, 5 min
- Remove the xylene with propanol for three changes.
- Wash in water for 15 sec.
- Stain in haematoxylin for 30-45 seconds.
- Wash in water
- Dip the smear in eosin stain for 15-30 seconds.
- Rinse in water for 15 seconds and remove excess of water.
- Dehydrate in three changes absolute propanol
- Clear in two changes xylene
- Mount in suitable synthetic resin (DPX)

RESULTS

- Nuclei, RNA rich cytoplasm, calcium Blue
- Muscle, fibrin, keratin Bright Red
- Bacteria Pink

(*Helicobacter pylori stain pink in color and identified by its Characteristic spiral or curved shape*)
(3) GIEMSA STAIN

- Remove paraffin wax with xylene, 30 minutes
- Prepare working Giemsa staining solution by mixing:
  - 1 part unshaken Giemsa stain
  - 9 parts buffered water.
- Flood the slide with working Giemsa
- Flow by pipettes and then put for 10-12 min.
- After 10-12 min, wash the slide and mount with DPX.
- Observed

Results

*Helicobacter pylori* stain blue in color and identified by its characteristic spiral or curved shape.
(3) WARTHIN-STARRY STAIN (Silver impregnation techniques)

PRINCIPLE

The method is based on the principle of silver impregnation. Slide with tissue section is dipped in silver nitrate solution in warm acidic condition this allows silver to impregnate on organism. After treatment with developer impregnated silver is converted into metallic silver which give dark brown appearance.

Preparation of reagents of Warthin-Starry stain

<table>
<thead>
<tr>
<th>Solutions:</th>
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</thead>
<tbody>
<tr>
<td>a) Acidulated water (pH 4.0)</td>
</tr>
<tr>
<td>b) Silver nitrate solution (AgNO₃) (Qualigenes Fine Chemicals, Mumbai)</td>
</tr>
<tr>
<td>c) 5% Gelatin (Hi Media, Mumbai)</td>
</tr>
<tr>
<td>d) 0.15% Hydroquinone (Merck Specialties Private Limited, Mumbai)</td>
</tr>
<tr>
<td>e) Developer (freshly prepared)</td>
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</tbody>
</table>
PROTOCOL

- Acidulate 1 liter distilled water with 0.1 g citric acid until pH of 4.0 is reached.
- Reagents for in house Warthin-Starry is recapitulate in Table I.
- Deparaffinised and Rehydrated sections were rinsed in distilled water and immersed in 1% AgNO₃ solution preheated in 50ºC water bath.
- Allow silver to impregnate the slide for 30 minutes, Meanwhile warm 2% AgNO₃, 5% gelatin and 0.15% hydroquinone at 54ºC water bath with closed lid(prevent oxidation), while the slide is impregnated, prepare the developer as shown in Table I.
- Remove the slides from the impregnator, and flood them with the warm developer until they show light brown or yellow color.
- Wash quickly with hot tap water (50ºC), rinse in distilled water, dehydrate in 95% alcohol (1 min), then absolute alcohol (10 sec), clear with xylene (2changes) by placing the slides in 2 jars for 5 min each. Mount with DPX.

Results

Organism appears characteristics brownish black against a light brown or yellow background.

(*Helicobacter pylori* identified by its characteristic spiral or curved shape)
6) DNA ISOLATION & POLYMERASE CHAIN REACTION (PCR)

DNA isolation from biopsy done by using “QIAamp DNA mini kit” from QIAGEN.

- Reagent preparation from QIAamp kit:-
  - Buffer AL: Mix thoroughly before use.
  - Buffer AW1: It is wash buffer. It is supplied as concentrate. So before using first time, add the appropriate amount of ethanol (96-100%).
  - Buffer AW2: It is wash buffer. It is supplied as concentrate. So before using first time, add the appropriate amount of ethanol (96-100%).
  - Buffer ATL: It is lysis buffer.
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- AE buffer

DNA ISOLATION PROCEDURE:

1. Excise the tissue sample or remove it from storage. Determine the amount of tissue. Do not more than 25mg.

2. Cut up to 25mg of tissue (up to 10mg) into small pieces. Place in 1.5 ml micro centrifuge tube, and add 180µl of buffer ATL. Proceed with step 3.

3. Add 20µl proteinase K, mix by vortexing, and incubate at 56°C until the tissue is completely lysed. Vortex occasionally during incubation to disperse the sample, or place in a shaking water bath or on a rocking platform.

4. Briefly centrifuge the 1.5ml micro centrifuge tube to remove drops from the inside of the lid.

5. Add 200µl Buffer AL to the sample, mix by pulse-vortexing for 15s, and incubate at 70°C for 10 min. Briefly centrifuge the 1.5ml micro centrifuge tube to remove drops from inside the lid.

6. Add 200µl ethanol (96-100%) to the sample, and mix by pulse vortexing for 15s. After mixing, briefly centrifuge the 1.5 ml micro centrifuge tube to remove drops from inside the lid.

7. Carefully apply the mixture from step 6 (including the precipitate) to the QIAamp Mini spin column (in a 2ml collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 × g (8000 rpm) for 1min. Place the QIAamp Mini spin column in a clean 2ml collection tube (provided), and discard the tube containing the filtrate.
8. Carefully open the QIAamp Mini spin column and add 500µl buffer AW1 without wetting the rim. Close the cap, and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2ml collection tube (provided), and discard the collection tube containing the filtrate.

9. Carefully open the QIAamp Mini spin column and add 500µl buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed (20,000 x g; 14,000 rpm) for 3 min.

10. Recommended: Place the QIAamp Mini spin column in a new 2ml collection tube and discard the old collection tube with a filtrate. Centrifuge at full speed for 1 min.

11. Place the QIAamp Mini spin column in a clean 1.5 ml microcentrifuge tube and discard the collection tube containing the filtrate. Carefully open the QIAamp Mini spin column and add 200µl Buffer AE or distilled water. Incubate at room temperature for 1 min. and then centrifuge at 6000 x g (8000 rpm) for 1 min.

12. Then repeat step 11.

POLYMERASE CHAIN REACTION :-

- Reagent preparation :-

1. 1X PCR buffer : 50mM KCL, 10mM tris HCL, 2.5mM MgCl2, 0.01% gelatin, 0.1% Triton X-100%
2. 0.2mM each dNTPs
3. 0.25U of Taq DNA Polymerase
4. Distilled water
5. PCR tubes

6. 100 Pico mole of each primer

\[
\begin{align*}
\bullet \ \text{cag} \ 1 & : 5' \ GAT \ AAC \ AGG \ CAA \ GCT \ TTT \ GAGG \ 3' \\
\bullet \ \text{cag} \ 2 & : 5' \ CTG \ CAA \ AAG \ ATT \ GTT \ TGG \ CAG \ A \ 3' \\
\bullet \ \text{vac} \ 1 & : 5' \ ATG \ GAA \ ATA \ CAA \ CAA \ ACA \ CAC \ 3' \\
\bullet \ \text{vac} \ 2 & : 5' \ CTG \ CTT \ GAA \ TGC \ GCC \ AAAC \ 3' \\
\bullet \ \text{ure} \ 1 & : 5' \ AAG \ CTT \ TTA \ GGG \ GTG \ TTA \ GGG \ GTTT3' \\
\bullet \ \text{ure} \ 2 & : 5' \ AAG \ CTT \ ACT \ TTC \ TAA \ CAC \ TAA \ CGC3' \\
\bullet \ \text{ice} \ 1 & : 5' \ TAT \ TTC \ TGG \ AAC \ TTG \ CGC \ AAC \ CTG \ AT \ 3' \\
\bullet \ \text{ice} \ 2 & : 5' \ GGC \ CTA \ CAA \ CCG \ CAT \ GGA \ TAT \ 3'
\end{align*}
\]

- Procedure

[1] The amplification of DNA was carried out in 0.2ml reaction tube by PCR using a thermal cycler (ESCO). A 50µl reaction mixture consist of
  - 5µl of 1X PCR buffer
  - 8µl of 0.2mM dNTPs
  - 0.5µl each primer
  - 0.6µl of Taq DNA polymerase
  - 25.4µl Distilled water

[2] Perform PCR according to the following program:-
**MATERIALS AND METHODS**

*cag A gene*

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Then store the amplified product at 2-4°C

Analysis of PCR Product by Agarose Gel electrophoresis

1. Prepare 2% agarose gel containing 1.5µl ethidium bromide in 0.5X TBE buffer.

2. Load 13µl of sample mixed with 2µl gel loading dye and 3µl 100bp molecular marker (B. Genie).

3. Run the gel at 100volts.

4. See the bands under transilluminary.