MATERIALS AND METHODS

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Chapter II

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ISOLATION OF HELICOBACTER PYLORI FROM GASTRIC BIOPSY SAMPLES

Collection of biopsy samples

The patients, who were pathologically (endoscopy) diagnosed for gastric cancer and peptic ulcer, were chosen for the collection of biopsy samples. These patients were the out patients of Baby Memorial Hospital, Calicut.

Transportation of specimen

Immediately after collection, the biopsies were transferred to sterile thioglycollate broth containing casein enzymatic hydrolysate (1.5%), dextrose (0.55%), sodium chloride (0.25%), L-cystein (0.05%), sodium thioglycollate (0.05%), Nalidixic acid (0.005%), and vancomycin (0.001%). The transportation to the laboratory was carried out at 4°C in a mini-cooler.

Processing of biopsy specimens

Rapid urease test (RUT)

All processing and cultures were performed in a vertical air laminar flow (Kirloskar Electrodyne Ltd.)

The biopsy specimens were divided into two halves. One part was used for RUT and the other part for isolation of H. pylori. The method of McNulty et al. (1989) was followed for RUT. One part of the biopsy from each patient was placed in a test tube with 1 ml. of sterile rapid urease test media containing 2% urea, 0.2% dipotassium hydrogen phosphate, 0.5% sodium chloride and 0.004% phenol red (pH 6.8). The tubes were incubated at room temperature up to 24 h and the results were characterised on the basis of the colour change of the medium. The biopsies were considered as strongly positive, moderately positive and weakly positive if the positive result
developed within 6, 12 and 24 h of incubation respectively. The results were noted as negative if there is no colour change produced even at 24 h of incubation.

Isolation of *H. pylori*

The second part of the biopsy sample of each patient was processed for isolation of *H. pylori* in culture. By using sterile forceps, the specimen was placed on a sterile microscopic slide and 2-3 drops of thioglycollate broth was added. The tissue was teased into fine spreads with forceps and needle. This was then streaked on chocolate agar plates containing 0.005% nalidixic acid, 0.001% vancomycin, and 0.005% trimethoprim. The plates were incubated either in a CO₂ incubator (ShelLab Model TC 2323) or in an anaerobic jar using gas-generating pack (Anaerocult C from EMERCK, Germany). The incubation was done at 37°C in an atmosphere containing 10% CO₂ and 95% humidity for 4 days. The unused tissue macerate on the slide was subjected to gram's staining. The photograph of the biopsy specimen showing the organism was taken using Leitz Dialux 20 Microscope with photographic facility.

Characterization of Isolates

Characteristics of each colony formed on the culture plates were noted and subjected to Gram's staining. Those colonies, which showed characteristic cell morphology of *H. pylori*, were selected and subculture made on fresh plates. The pure cultures of each isolate were used for the following biochemical characterization.

a) Catalase test: The method of Taylor and Achanzar (1972) was adopted. A small portion of the colony was picked with a sterile capillary tube and immersed in H₂O₂ solution (10% v/v) in a tube. The generation of effervescence noted as positive reaction.

b) Oxidase test: The method reported by Steel (1961) was adopted. The isolate was taken with the sterile capillary tube and rubbed on a filter paper impregnated with 1% solution of NNNN'-tetra methyl para phenylene diamine dihydrochloride. The development of purple colour within 10 s is considered as positive reaction.
c) Urease test: The test was performed on Christensen's urease medium (Christensen, 1946). The media was prepared and the colonies were inoculated on to the slants. Incubation was carried out in a CO₂ incubator with 10% CO₂ at 37°C. The colour change of the media from golden yellow to pink was noted as positive reaction.

The cultures were also tested for its ability to utilize glucose and nitrate reduction. Motility of the organism was tested.

The biochemical characters expressed by the colonies were compared with the characteristics of *H. pylori* reported in standard references. The characters were also compared with those of a standard strain (HP 101) obtained from Dr. A. Grob (Germany).

ANALYSIS OF ANTI-*H. PYLORI* IgG AND IgG SUBCLASSES IN GASTRIC CANCER PATIENT SERA

Collection of serum samples

Serum from gastric cancer patients, who were the inpatients of Regional Cancer Centre, Thiruvananthapuram or Medical College Hospital, Calicut, was collected for the analysis of anti-*H. pylori* IgG and IgG subclasses. Only those cases, which were diagnosed as gastric cancer, were selected. Patients at different age group from 24-75 were included in the present study. Age matched healthy persons were included as controls.

5 ml Blood from each patient was taken by venipuncture and allowed to clot and serum was separated by centrifuging at 900 g for 10 min in a tabletop centrifuge. The separated serum was stored at ~20°C in a freezer until used for the assay.

Anti-*H. pylori* IgG Analysis

The level of anti-*H. pylori* IgG in the serum samples were analysed by using commercially available diagnostic kits (UBI Magiwel EIA, United Biotech Inc., Mountain
The assay was done as described below. 100 μl of serum samples at 1:101 dilutions was added to pre-coated wells with H. pylori antigens. Calibrator, positive control and blank were maintained in the assay. The plates incubated at room temperature for 30 min. After the incubation, the wells were washed five times with wash buffer. 100 μl of enzyme conjugate (anti-human IgG-HRP conjugate) was added to each well and the plates were incubated for 30 min. After washing the plate, 100 μl of substrate with chromogen was added to the wells. The colour was developed by incubating the plates for 15 min at room temperature. 50 μl of 1 N \( \text{H}_2\text{SO}_4 \) was added to the wells to stop the reaction and the plates were read in an ELISA reader at 450nm.

The amount of anti-\( H. \text{pylori} \) IgG in each patient sample was calculated by using the formula given by the manufacturer

\[
\text{Anti-}H. \text{pylori} \text{ IgG (EU/ml sample)} = \frac{\text{O.D. of the test sample}}{\text{O.D. of the calibrator}} \times \text{EU/ml of calibrator}
\]

The samples were considered positive for \( H. \text{pylori} \) antibodies if the sample contains 40 or above EU/ml.

**Analysis of IgG subclasses in serum samples**

Purified monoclonal antibodies specific to human IgG subclasses, such as HG-11 for IgG\(_1\) (Mayus et al., 1986), Hp-6016 for IgG\(_2\), Hp-6066 for IgG\(_3\), and Hp-6011 for IgG\(_4\) were used for coating the micro titre plate. Specific myeloma proteins W-6, W-238, W-242 and W-210 for IgG\(_1\), IgG\(_2\), IgG\(_3\) and IgG\(_4\) respectively were used as subclass specific standards and WHO 67/97, a pooled serum assigned for IgG subclasses, was used as reference serum sample. All the above reagents were a gift from Dr. M. H. Nahm, Washington University, School of Medicine, St. Louis, Missouri, U.S.A. Goat anti-human IgG peroxidase conjugate was a gift from Immunological Lab, National Institute of Immunology, New Delhi.
Micro titre plates were purchased from Laxbro, product of Bhagawandas and Company at Pune. Bovine serum albumin (BSA), and o-phenylene diamine dihydrochloride (OPD) were purchased from Sigma chemicals, St. Louis, U. S. A. All other reagents used in the assay were purchased from MERCK QUALIGENS, India.

Optimization of ELISA System

The method of Butch et al. (1989) was adopted as the basis for optimisation of ELISA system. 5, 10, and 20 µg/ml concentrations of subclass specific monoclonal antibodies were prepared in 0.1 M carbonate buffer (pH 9.6) and 150 µl of each concentration was used for coating micro titre plates. The experiments were done in quadruplicates. The plates were incubated at room temperature for 3 h followed by washing with 0.05% Tween-20 in 10 mM PBS (pH 7.2). The wells were blocked with 200 µl of 1% BSA in PBS with 0.05% Tween-20 for 30 min. The plates were washed and 150 µl of appropriately diluted serum samples, pooled normal serum (n=50) and WHO-67/97 reference serum (as quality control) were added into different wells. 150 µl of subclass specific myeloma proteins ranging from 2-100 ng concentrations were used as standards. The plates were incubated at room temperature for 3 h. Washing step was repeated. 150 µl of different dilutions of goat-antihuman HRP conjugate (1:2500, 1:5000, 1:10000) were added to the wells as checkerboard and incubated further 3 h at room temperature. Washed the plates and 150 µl of the substrate (0.04% OPD in 0.1 M citrate buffer, pH 5 mixed with H2O2) was added and kept in dark at room temperature for 20 min. The reaction was stopped by addition of 75 µl of 1N H2SO4 to each well.

The plates were read in an ELISA reader (Biotech Model ELx 800, Biotech Instruments Inc.) On the basis of data obtained it was decided to choose 10 µg/ml subclass specific monoclonal antibodies and 1:5000 dilution of secondary antibody conjugates for the analysis of IgG subclasses.
ELISA for IgG Subclasses

150 µls of 10 µg/ml solutions of HG-11, Hp 6016, Hp 6066, and Hp 6011 were used to coat the micro titre plate for IgG1, IgG2, IgG3 and IgG4 respectively. Serum samples were diluted to 1:40000 for IgG1, 1:20000 for IgG2, 1:5000 for IgG3 and 1:100 for IgG4 with 1% BSA in 10 mM PBS (pH 7.2). 225 µl of each dilution of the serum sample were added to the first wells of the plates for IgG1, IgG2, IgG3 and IgG4 assay. It was then serially diluted 3-fold in the subsequent wells up to 6 wells i.e., 75 µl from the first well was taken and transferred to the next well containing 150 µl of BSA solution with the help of a micropipette (Glaxo, Germany). 150 µl of 1:5000 dilution of secondary antibody enzyme conjugate was added to each well. All other steps were same as described in optimisation of ELISA. In each plate pooled serum samples, standards for respective subclasses and WHO-67/97 reference serum were included as quality controls.

The concentration of each subclass in the sample was determined from standard graph plotted in the semi-log paper by comparison to the optical density generated by standard proteins. The sensitivity of the assay was 2 ng/ml.

PREPARATION OF ANTIGENS FROM H. pylori STRAINS (ISOLATES)

Different isolates of H. pylori were grown on chocolate agar containing antibiotic supplement. After incubation for 48 h in microaerophilic conditions, the cells were harvested by scraping and were washed twice with PBS (0.1 M, pH 7.2) by centrifugation at 8000 rpm for 15 min at 4°C in a refrigerated centrifuge (Plastocraft Superspin-R model) and various methods used for the preparation of antigens.

Acid-Glycine Extraction (AG)

The method described by Hirschl et al. (1988) was followed with some modifications. 2.5 ml of 0.2 M glycine-HCl buffer (pH 2.2) was added to 100 mg (wet weight) H. pylori cells. The suspension was mixed gently at room temperature for 20 min. It was then centrifuged at 15000 rpm for 15 min at 4°C in a refrigerated centrifuge.
The supematant was neutralized by adding 1 M NaOH and dialyzed against PBS for 24 h at 4°C. Particles, if any, were centrifuged out and the preparation was lyophilised in a Savant lyophilizer (RVT 400 model) and stored at −80°C deep freezer (NUAIRE, NU2500E).

**n-Octyl-β-D-Glucopyranoside Extraction (NOG)**

The method of Evans Jr. et al. (1989) was adopted for n-octyl-β-D-glucopyranoside extraction of antigens from *H. pylori*. The cell suspension was centrifuged at 8000 rpm for 15 min at 4°C. The pellet was suspended in 2.5 ml of 1% solution of n-octyl-β-D glucopyranoside (Sigma Chemicals, St. Louis, USA) in sterile PBS (pH 7.2). After incubation for 20 min at room temperature, the suspension was centrifuged at 12000 rpm for 15 min at 4°C. The extract was dialysed against half concentrated PBS (0.05 M, pH 7.2) at 4°C followed by dialysis against distilled water at 4°C. The particles, if any, were centrifuged out and the supernatant lyophilised and stored at −80°C in a deep freezer.

**Whole Cell Sonicate Preparation (WCS)**

Method described by Hirschl et al. (1988) was followed. The cell suspension was washed twice in PBS and it was inactivated at 60°C for 15 min. The inactivated cells were resuspended in PBS and sonicated 4 x 10 seconds by keeping in an icebox. The sonicate was centrifuged at 12000 rpm for 20 min and the supernatant concentrated by using a lyophilizer (Savant, RVT 400 model). The preparation was stored at −80°C in a deep freezer till used for further experiments.

**Hot Phenol-Water Extraction (LPS)**

The method of Westphal and Khan (1965) was adopted for hot phenol-water extraction. The cells from 25 plates were harvested into PBS and washed as described earlier. The pellet was resuspended in water and an equal amount of 90% phenol (pre-heated to 65-68°C) was added. The mixture was vigorously shaken and kept at 65°C. After incubation for 15 min it was cooled to 10°C and centrifuged at 12000 rpm
for 20 min. The water layer was collected and the extraction procedure was repeated twice. The water-extracted layers were pooled and dialysed against distilled water to remove any residual phenol (48 h at room temperature with four changes of water). Suspended particles, if any, were removed by centrifugation at 12000 rpm for 10 min. The supernatant lyophilised and stored at -80°C in a deep freezer.

**Sodiumdodecyl sulphate-Polyacrylamide gel Electrophoresis (SDS-PAGE)**

Protein profile of each antigen preparations, except hot phenol-water extract, was determined by SDS-PAGE. The method of Laemli (1970) was adopted. The separation was carried out in a 10.5% separating gel with 5% stacking gel. For preparing separating gel 3.03 ml double distilled water, 2.25 ml 1.5 M Tris (pH 8.8), 0.9 ml 10% SDS, 0.46 ml glycerol and 3.15 ml 30% Acrylamide-0.8% bis-acrylamide were mixed. Before casting the gel 6 µl of TEMED, and 60 µl of 10% ammonium per sulphate solutions were added to the mixture.

After polymerisation, the gel was washed thrice with distilled water. The stacking gel was prepared by mixing 1 ml 0.5 M Tris (pH 6.8), 40 µl of 10% SDS, 667 µl of 30% acrylamide-0.8% bis-acrylamide, 2.24 ml double distilled water, 4 µl TEMED, and 53 µl of 10% ammonium per sulphate. A comb was placed and the stacking gel was prepared. 100 µl of each sample (containing 100 µg protein) was mixed with 50 µl of sample buffer containing SDS, glycerol, EDTA, 0.5 M Tris (pH 6.8) and bromophenol blue in double distilled water. The tubes were kept in boiling water bath for 3 min. The wells of the gels washed thrice with running buffer (Tris-glycine, pH 8.3) and 25 µl of the sample was added to each well. Molecular weight markers (Medium range, purchased from Bangalore Genei Pvt. Ltd.) were also included in the experimental protocol. The electrophoresis was carried out in BioRad Mini Gel Unit (BioRad, Hong Kong) at a constant voltage (100 V) using Hoeffer power supply (Hoeffer Scientific Instruments, Sanfrancisco, California). After the electrophoresis the gels were stained
with Coomassie Brilliant Blue- R250 (BioRad) followed by destaining with a mixture of methanol, acetic acid, and water.

**IN VITRO SECRETION OF IgG SUBCLASSES**

**Isolation of mononuclear cells from tonsils**

Fresh human tonsils were chosen for obtaining mononuclear cells for the present study because it will contain 60-70% B-cells, 30-40% T-cells, 1-8% monocytes and <1% dendritic cells. Thus a combination of all cells required for adequate humoral response will be available for in vitro studies.

Immediately after tonsillectomy, the specimens were collected and transported to the laboratory at 4°C. The processing of the specimens was done within 2 h of collection.

**Processing of the tonsils**

Protocol described by Coligan et al. (1996) was followed. Briefly, the specimens were placed on a sterile stainless steel sieve, which was set on a sterile petridish. The tissue was cut into 3-5 mm pieces and squeezed through the mesh using flat end of a syringe plunger.

The cell suspension was transferred to 50 ml sterile centrifuge tubes and allowed to stand 5 min. The debris was removed. The suspension over-layered on Ficol-Hypaque gradient (density 1.007 g/L) (Pharmacia Biotech, Upsala, Sweden) and centrifuged at 900 g for 20 min at room temperature. The layer of mononuclear cells (Buffy coat) at the interface was collected and transferred to fresh sterile centrifuge tube. The cells were washed three times with sterile PBS. The final washing was done with RPMI medium.

The cell pellet was re-suspended in complete RPMI medium and checked the viability with trypan blue as described by Bortran and Vetvika (1995) and counted the number of cells with a haemocytometer. The cell density was adjusted to 2 x 10^6 cells/ml.
Proliferation assay

200 µl of the above cell suspension was dispensed into each well of a 48 well culture plate (Costar, Sigma, St. Luis, USA). Con A (purchased from CSIR Centre for Biochemicals, New Delhi) and PHA (purchased from HiMedia laboratories, Mumbai) were used as mitogens. Different concentrations of mitogens, such as 10, 20, 30, 40, 50, 60, 70, and 80 µg/ml were added to the wells. Similarly, different concentrations of H. pylori antigens obtained from various preparations were also added to each well. Control wells were also included in the protocol without mitogens or antigens. The plates were incubated at 37°C at 5% CO₂ atmosphere and 95% humidity (NUAIRE, IR auto flow-water jacketed) for 72 h.

The proliferation of cells in cultures was assayed by using 3-(4,5-dimethyl-2-thioazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (Mosman, 1983). At the end of incubation, 20 µl of sterile MTT solution (5 mg/ml in PBS) was added to each well and incubated for 4 h at 37°C followed by the addition of 200 µl of acidic isopropanol (0.04 N HCl in Isopropanol) to each well and kept at room temperature to dissolve all crystals formed (5-10 min). After mixing the content of the wells, optical density was measured at 570 nm in a Spectrophotometer (Shimadsu, UV 1601 model) with 0.5 ml cuvettes.

Assay of IgG subclasses

The cultures were prepared as described above. After incubation for 7 days, the level of IgG subclasses in the culture soup was assayed by following ELISA technique (Butch et al., 1989). The protocol was same as that described in the assay of IgG subclasses in serum samples, except for the sample dilution. The culture soup was diluted 2 times for estimating the IgG subclasses. The level of each subclass was determined from the standard graph generated for each subclass.
MACROPHAGE ACTIVATION STUDIES

Collection of murine peritoneal exudates cells (PEC)

Peritoneal cavity of mice provides an accessible site for the harvest of resident macrophages. Generally the normal mouse peritoneal cavity will yield mature, resident macrophages.

The protocol described by Coligan et al., 1996) was adopted. The mice were killed by cervical dislocation. Making an incision followed by peeling off the skin was performed to expose the abdominal wall. 5 ml complete RPMI medium containing 5 µg/ml heparin was injected into the peritoneal cavity and the injected medium was circulated into the cavity by gentle massage. The medium was aspirated with the help of a syringe and collected into 15 ml centrifuge tubes. The suspension was centrifuged at 4000 rpm for 15 min and the pellet was washed with RPMI medium and re-suspended in complete RPMI medium. The viability of the cells was checked with trypan blue. The cell numbers were counted by using a haemocytometer and the cell density was adjusted to $1 \times 10^6$ cells/ml.

100 µl of PEC suspension was dispensed in each well of a 96 well culture plate (TARSONS Products Pvt. Ltd. Kolkata) and placed in a humid CO$_2$ incubator for 3 h at 37°C. The medium was removed and the adherent cells were washed with RPMI medium. The wells without cell suspension and only with CRPMI medium served as controls. 100 µl of different concentrations of antigens (5, 10, 20, 30, 40, and 50 µg/ml) and mitogens (5, 10, 20, 30, 40, and 50 µg/ml) in complete RPMI medium were added into respective wells. Each concentration was prepared in triplicates. The plates were incubated at 37°C in an incubator with 5% CO$_2$ and 95% humidity.

Assay of nitric oxide (NO)

The method described by Coligan et al., 1996) was adopted. 200 µl culture supernatant was mixed with 200 µl Griess reagent (an equal mixture of
1% sulphanilamide in 5% orthophosphoric acid and 0.1% naphthyl ethylene diamine in distilled water) in separate tubes and incubated at room temperature for 20 min.

Standard was prepared by taking sodium nitrite at the range of 50-500 ng in CRPMI medium and treated similar to that of test samples. The blank was included in the experimental protocol. The optical density of the colour developed was measured at 540 nm in a spectrophotometer with 0.5 ml cuvettes.

Standard graph was plotted and the concentration of NO\textsuperscript{2−} in the sample was read from the graph. The amount of NO formed was expressed in nM/10\textsuperscript{6} cells/24 h.

Release of enzymes from Macrophages

After removing culture soup from each well, the adherent macrophages were washed three times with sterile normal saline. 50 µl of cold double distilled water was dispensed into each wells and kept at 4°C overnight. The cell lysate was taken in the eppendorff tubes and centrifuged at 10000 rpm for 10 min at 4°C. The supernatant was used for the enzyme assay.

Assay of Acid Phosphatase

The method described by Burch (1969) was adopted with following modifications. Briefly, 100 µl of acetate buffer (0.1 M, pH 5) and 30 µl of double distilled water were dispensed into each well of a micro titre plate. 40 µl of the enzyme preparation from the macrophages was added to the wells. Mixed well and 25 µl substrate (p-Nitro phenyl phosphate - 0.05 M) was added to all wells, except control wells. The plates were incubated for 1 h at 37°C in a humid chamber. After incubation, the reaction was stopped by adding 40 µl of 6 N NaOH to each well, including control wells. 25 µl substrate was added to the control wells. The experiments were conducted in duplicates. The intensity of colour developed was measured at 420 nm in a Spectrophotometer with 0.5 ml cuvettes.

A standard graph was prepared by using different concentrations of p-Nitrophenol as standard. The level of enzyme activity in presence of different antigen
preparations and mitogens were read from the standard graph and expressed as nM of p-Nitrophenol formed/10^6 cells/h.

**Assay of β-Glucuronidase**

The method described by Fishman and Bernfeld (1968) was adopted with the following modifications. Briefly, 25 µl acetate buffer (0.1 M, pH 5) was dispensed into each wells of a micro titre plate. 50 µl of enzyme preparation from macrophages was added to the wells in duplicates. 25 µl substrate (Phenolphthalein glucouronic acid - 1 mg/ml) was added to each well, except in control wells. The plate was incubated at 37°C in a humid chamber for 1 h. The reactions was stopped by adding 100 µl of stopmixture, which contain 1.503 g glycine, 5.299 g sodium carbonate, and 0.6 g sodium hydroxide in 100 ml distilled water, including in control wells. 25 µl of substrate was added to control wells. The intensity of the colour developed was measured at 540 nm in a spectrophotometer with 0.5 ml cuvettes.

A standard graph was prepared by using different concentrations of phenolphthalein as standard. The enzyme activity in the presence of antigen preparations and mitogens were read from the standard graph and expressed as nM phenolphthalein formed/10^6 cells/h.

**ANALYSIS OF LIPID PROFILE**

**Estimation of total cholesterol in serum samples (Cholesterol oxidase-Peroxidase method)**

Commercially available diagnostic kits purchased from Span diagnostics, Surat, India were used to assess the total cholesterol present in the serum sample of patients. 10 µl of serum sample and 10 µl of standard were added to 1 ml of cholesterol reagent in separate tubes. All tubes were mixed well and incubated at 37°C for 10 min. The optical density of the colour developed by samples and standards were measured against reagent blank, which contain only cholesterol reagent, at 505 nm using a UV-Visible spectrophotometer (Shimadsu, UV1601 model).
The method is based on the following enzymatic reactions:

\[
\text{Cholesterol esters} \xrightarrow{\text{Cholesterol esterase}} \text{Cholesterol} + \text{Fatty acids}
\]

\[
\text{Cholesterol} + O_2 \xrightarrow{\text{Cholesterol oxidase}} \text{Cholesten-3-one} + H_2O_2
\]

\[
H_2O_2 + 4\text{-AAP} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine} + H_2O
\]

The amount of total cholesterol in the samples was calculated by using the following formula.

\[
\text{Total cholesterol (mg/dL)} = \frac{\text{Absorbance of the test} \times 200}{\text{Absorbance of the standard}}
\]

**Estimation of HDL-cholesterol**

Commercially available diagnostic kits purchased from Span diagnostics, Surat, India were used for the estimation of HDL-cholesterol. 0.3 ml of the precipitating reagent (Polyethylene glycol 6000) was mixed with 0.3 ml of the sample. After incubating for 10 min in room temperature, the tubes were centrifuged at 4000 rpm for 10 min and the clear supernatant was collected. 1ml of cholesterol reagent was added to 100 µl of the supernatant and 100 µl of the HDL standard and incubated at 37°C for 10 min. The optical density of the colour developed was measured at 505 nm in a spectrophotometer against the reagent blank, which contain only cholesterol reagent.

The level of HDL cholesterol was calculated by using the following formula.

\[
\text{HDL Cholesterol (mg/dL)} = \frac{\text{Absorbance of the test} \times 100}{\text{Absorbance of standard}}
\]

**Estimation of triglycerides**

Commercially available diagnostic kits purchased from Span diagnostics, Surat, India was used for the estimation of triglycerides in patient sera. Manufacturer's instructions were followed to perform the test. 10 µl of sample and 10 µl of triglyceride standard were added to 1 ml of triglyceride reagent in separate tubes. Incubated at 37°C for 10 min. The optical density of the colour developed was measured at 505 nm.
in spectrophotometer against the reagent blank, which contain 1 ml triglyceride reagent only.

The method of estimation is based on the following enzymatic reactions

\[
\text{Triglycerides} \xrightarrow{\text{Lipoproteinlipase}} \text{Glycerol + Free fatty acids}
\]

\[
\text{Glycerol + ATP} \xrightarrow{\text{Glycerokinase}} \text{Glycerol-3-phosphate + ADP}
\]

\[
\text{Glycerol-3-phosphate} + \text{O}_2 \xrightarrow{\text{Glycerol-3-phosphatase}} \text{Dihydroxyacetone phosphate + H}_2\text{O}
\]

\[
2\text{H}_2\text{O}_2 + 4\text{- AAP} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine + H}_2\text{O}
\]

The amount of triglycerides in the samples was calculated by using the formula.

\[
\text{Total triglycerides (mg/dL)} = \frac{\text{Absorbance of the test} \times 200}{\text{Absorbance of the standard}}
\]

Estimation of very low-density lipoprotein (VLDL) and low-density lipoprotein (LDL)

The formula derived by Friedewald et al. (1972) was used to calculate cholesterol in VLDL and LDL fractions in serum samples.

\[
\text{Cholesterol in VLDL fraction} = \frac{\text{Total triglycerides}}{5}
\]

\[
\text{Cholesterol in LDL fraction} = \text{Total cholesterol} - \left[\frac{\text{Total triglycerides}}{5} - \text{HDL}\right]
\]