CHAPTER-II

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MATERIALS & METHODS

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Materials:

1. **Microlab 200 Auto Analyzer:** For all bio-chemical tests auto analyzer – 'microlab-200' (MERCK) was used. It has six filters starting from 340 nm to 620 nm. It has memory for 60 programs. It has capacity to perform all end points chemistries, all Kinetic chemistries can plot and store multi-standard graphs. Aspiration device can be programmed to aspirate as low a volume as 300 μl. For end point chemistry one point calibration was done. For Kinetic chemistries factors were fed as per product insert of various kits.

2. **Neuber's Chamber:** For counting of RBC's and WBC's Neuber's Chamber was used.

3. **Micropipetts:** Fixed volume auto pipetts were used in series, volumes used were (a) 5 micro liters, (b) 10 micro liters, (c) 50 micro liters, (d) 100 micro liters and (e) 1000 micro liters.

4. **Centrifuge:** Remi – R8C Model to accommodate 16 tubes at a time. It is used for blood contrifugation.

5. **Incubator:** YORCO make Incubator (Scientific Industries) was used for incubation.

6. **Hemoglobin Kit:** It is based on cyanmethemoglobin method (Qualigens). The standard provided with the kit has a value of 15 gm/dl.

7. **Glucose:** (Span diagnostic) 9 X 50 ml pack was used. Standard provided has a value of 100 mg/dl.

8. **Protein:** (Span diagnostic) 1 X 100 ml pack was used. Standard provided has a value of 6.5 gm/dl.

9. **SGOT:** (E. Merk India Limited) Liquid stable based on reference method of International Federation of Clinical Chemistry (IFCC).

10. **SGPT:** (E. Merk India Limited) Liquid stable based on reference method of International Federation of Clinical Chemistry (IFCC).

11. **Acid Phosphatase:** (Clonital – Italy – Teco Diagnostics) Total Acid Phosphatase was taken into consideration.

12. **LDH:** Teco diagnostic based on IFCC. Reconstituted reagent has good stability.

13. **Quality Control Sera:** (Randox UK) Level II Control Sera was used in this study which has results in abnormal range having parameter. For comparision average data of various instruments were used.

14. **EDTA ULB:** Commercially available polystyrene vials containing tripot salt of EDTA were used.

15. Dispovan Disposable Syringes used.

Methods:

COLLECTION OF BLOOD: Blood samples of patients suffering from lung diseases were collected from District Government Hospital, Bilaspur (C.G.).

Blood samples were collected from the normal subjects and clinical patients afflicted with various lung diseases such as Asthma, Tuberculosis, Bronchi ectasis and Emphysema by applying routine methods.

Capillary or Venous blood was used for all determinations. The blood has been taken from a vein on the front of the elbow or fore arm. Swipe cleaned the skin over the vein with rectified spirit. A well-sharpened disposable syringe of 5 ml (make Dispovan) was to be carried out on a single person. It was inserted into the vein, which could be held steady by the thumb of other hand of the operator. When the needle entered the plunger was withdrawn slightly. When 3 ml of blood had been drawn into the syringe, a small pad of cotton soaked with spirit was placed on the arm where the needle was inserted and the needle was withdrawn, with a pad kept pressed until bleeding stopped.

The needle was removed from the syringe and the blood transferred into a closed container with an anti coagulant (5% EDTA) for Hematological determination.

For bio-chemical analysis serum was separated from the clotted blood. This was collected in a centrifuge tube and allowed the blood to clot for 15 minutes at room temperature. Remove the clot that was adhering to the wall of the centrifuge tube to dislodge the clot from the wall. Centrifuged at 2500 rpm for 5 minutes in a Remi-R8C model centrifuge. Separate the serum within 2 hours from the time of blood collection and keep the serum in labeled test tubes at room temperature or in ice.

HEMOGLOBIN ESTIMATION

Hemoglobin was determined in 'six' individual patients of Asthma, Tuberculosis and 'four' cases of Bronchiectasis and Emphysema as well six control subjects. Hemoglobin was determined by the cyanmethemoglobin method. The method is based on conversion of Hemoglobin to methemoglobin by Ferri cyanide and subsequent cyanide-based conversion of methemoglobin to cyanmethemoglobin which absorbs strongly at 540 nm. The absorbance is proportional to the Hemoglobin concentration.

Concentrated Stock Drabkin's Solution was diluted 20 fold with distilled water for making working Drabkin's solution. Drabkin's solution (5 ml) was taken in a clean dry labeled test tube and to it 20 µl of well mixed EDTA blood was added. After mixing properly kept at room temperature for 5 mins. Reading was taken at 540 nm in Microlab 200 Analyser against water as blank. Hemoglobin concentration has been calculated by factor using commercially available standard.

Factor = concentration of std/A 540 nm of std.
Concentration of sample = A 540 nm X Factor
Units = g/l

Calibration: Standard given in the kit was used to calibrate the Microlab 200 Analyzer. The instrument uses single point calibration method and calculates a factor (concentration of std. / A 540 nm of std.). The factor is directly computed by the Auto analyser and the result is displayed directly on the screen.

Quality Control: For every run at least 3 samples were run together which were earlier analyzed by Automatic Hematology Analyser (ODEN-160-E Merk India Limited). Good correlation was observed in all samples used as controls.

RBC NUMBER

Method: The RBC number was determined in the blood of control and patients of lung diseases. RBC was determined in six Asthma, Tuberculosis and four of Bronchi ectasis and Emphysema patients as well six control subjects.

The RBC number was made with Nuebauer Crystalline Counting Chamber. Concentration of RBC dilution fluid (Nova Biotech dilution) is dissolved in 200 ml of distilled water and kept at room temperature. It is violet in colour.

To 3.8 ml of making RBC solution 20 µl of well mixed EDTA blood is added (1:200 ratio) then it was turned into red in colour. After mixing properly it is kept at room temperature for 10 mins. The RBC number was represented in millions per cubic millimeter (mm^3). Diluted blood was taken up to '0' mark in the RBC pipette and was mixed thoroughly by rotating the pipette and the mixture was allowed to stand about 2-3 mins for uniform mixing. The counting chamber and cover glass were cleaned and the cover glass was placed over the portified area. Again, the solution was mixed gently and the stem full of solution was expelled and a drop of fluid was allowed to flow under cover slip, handling the pipette at an angle of 90 degrees. It was allowed to stand for 2 to 3 mins till the RBC settled. Afterwards the portified area of the counting chamber was focussed under the microscope (Lebomed) and number of RBC were counted in five small squares of the RBC columns. The RBC were counted in the four corner squares and the central square under high power. The number of RBC per square millimeter (mm2) was calculated using the following formula:

(Number of cells X dilution factor 200 X depth factor) / Area counted

= x million / mm^3

WBC NUMBER

Method: The WBC number was determined in the blood of control and patients of lung diseases. WBC was determined in six Asthma, six Tuberculosis and four of each Bronchiectasis and Emphysema and from six control subjects.

Concentrated WBC is dissolved in 20 ml of distilled water and mixed well kept at room temperature. To 3.8 ml of making WBC solution (Balk solution) (violet in colour) 20 µl of well mixed EDTA blood was added. Then it turned into red in colour. After mixing kept at room temperature for 10 mins the WBC number was represented in cells/cubic millimeter (mm^3).

Diluted blood was taken upto 0.5' mark in the WBC pipette and it was mixed thoroughly by rotating the pipette and mixture was allowed to stand about 2-3 mins for uniform mixing. The counting chamber and cover glass were cleaned and the cover glass was placed over the portified area. Again the solution was mixed gently and the stem full of solution was expelled and a drop of fluid was allowed to flow under cover slip, handling the pipette at an angle of 90 degrees. It was allowed to stand for 2-3 min till the WBC settled. Afterwards, the portified area of the counting chamber was focussed under the microscope (Lebomed) and number of WBC was counted in four big squares at the four corners under high power. The number of WBC per square millimeter (mm^2) are calculated using the following formula.

(Number of cells X dilution factor 20 X depth factor) / Area counted

= x cells / mm^3.

ESTIMATION OF BLOOD GLUCOSE

Blood glucose was estimated in both control and various lung – disease patients. The blood from individual person was collected in test tubes by disposable syringe and centrifuged to separate serum for bio-chemical assays. From each test tube 10 µl of blood serum was taken with a micro pipette and the glucose concentration in the blood, serum was determined by the method detailed below. Glucose content was expressed as mg of glucose per 100 ml of blood serum.

Method: Glucose Oxidase Peroxidase (GOD – POD). This procedure is based on conversion of glucose into gluconic acid by glucose oxidase and subsequent peroxidase catalyzed reaction. The liberated oxygen is trapped by the chromogen system to give a red colour quinoneamine compound. The colour developed is measured at 540 nm and is directly proportional to glucose concentration.

Reagent preparation: Hypo-Chloride reagent (50 ml) was added to each vial of lyophilyzed enzymes. The reagent thus reconstituted was stored at 28^0C . The reagent is stable up to 30 days.

Procedure: Working reagent (1 ml) was taken in a clean dry-labeled test tube with the help of micro pipette. To which serum (10 µl) was next added. After mixing it was incubated at 37^0C for 15 min. A light pink colour developed at end of incubation that was measured at 540 nm an Auto analyser against reagent blank.

Calibration: Single point calibration is done using glucose standard (Dextrose, Benzoic Acid) 1 mg/ml provided in the kit.

Quality Control: With every series level 'II Randox' control sera have been used. The values were within the control values.

ESTIMATION OF SERUM PROTEIN

Serum total protein was estimated in both control and patients of lung diseases. The blood from individual was collected into clean labeled test tubes with disposable syringe and centrifuged to separate serum for protein assays. Suitable aliquot (20 µl) of blood serum pipeted and the protein content in the serum was determined by the Burette method. Protein content was expressed as grams of total blood serum protein/ 100 dl of blood serum.

This calorimetric procedure is based measurement of peptide bonds of proteins is a coloured chelate formed with cupric ions in alkaline solution. The coloured product absorbs strongly at 546 nm. A 546 nm values are directly proportional to protein concentration.

Reagent preparation: Auto span Burette reagent kit contains ready to use four reagents and was used for the assays.

Procedure: With a micro pipette 1 ml of reagent was taken in all clean dry labeled test tubes, and to it 20 µl serum was added. Test tubes were incubated at 37^0C for 5 mins. After the end of 5 min a light violet colour was developed which was measured in Auto Analyser at 546 nm wavelength against reagent blank.

Calibration: Calibration was done as per standard protocol enclosed in the reagent kit.

Quality Control: With every series level 'II Randox' control sera have been used. The values were within the control value.

ESTIMATION OF SGOT (ASAT)

Serum Glutamate Oxalo Amino Transferase (ASAT) was estimated in serum obtained from control subjects and patients. Suitable aliquots of serum 50 ml were taken with a micro pipette and the SGOT content in the serum was determined by a U.V. Kinetic method. It is based on the reference method of International Federation of Clinical Chemistry (IFCC).

This procedure is based on conversion of Oxoglutarate into Glutamate and Oxalacetate by ASAT and oxalacetate reacted with NAD_2H oxidise the NAD^+. The rate of NADH consumption is measured and is directly proportional to the ASAT activity in sample.

Reagents: Reagents used were from Merck.

Procedure: To prepare reaction solution, mixed reagent 1 and 2 in 4 + 1 proportion. Reagent solution (400 ml) was mixed with 100 µl of start reagent. This solution (500 ml) was taken in a test tube and kept for 5 mins at 37^0C To it 50 µl serum was added. Absorbance was measured at 340 nm in a Auto Analyzer against distilled water. The reaction mixture was aspirated immediately. The instrument was programmed at 60 second delay to measure fall in Absorbance at 340 nm. Rate of change of OD/Unit time is calculated (A) which was multiplied with factor. Final result as well as graphic progress of the reaction was displayed on screen.

Calibration: Calibration of the instrument was done as per standard fixed operation procedure.

ESTIMATION OF SGPT (ALAT)

Serum Glutamate Alanine Amino Transferase (ALAT) was estimated in serum obtained from control subjects and patients. The blood from individual was collected into test tubes and centrifuged to separate serum for SGPT estimations. Suitable aliquots (50μl) of serum were taken with a micro pipette and the SGPT content in the serum was estimated by the U.V. Kinetic method. It is based on the reference method of International Federation of Clinical Chemistry (IFCC).

This procedure is based on conversion of amino group of Alanine into Glutamate and Pyruvate catalysed by Alanine Transferase. Pyruvate next reacts with NADH and oxidized it into NAD which absorbance strongly at 340 nm.

Reagents: Reagents used were from Merk.

Procedure: To prepare reaction solution, mixed reagent 1 and 2 in 4+1 proportion. Reagent solution (400 μl) mixed with 100 μl of start reagent. This solution (500 μl) was pipeted in a test tube and kept for 5 min at 370C degree centigrade temperature. To it 50 μl serum was added. Absorbance was measured at 340 nm in an Auto Analyzer against distilled water. The reaction mixture was aspirated immediately. The instrument was programmed at 60 second delay to measure fall in A 340 nm. Rate of change of OD/Unit time is calculated (A) which was multiplied with factor. Final result as well as graphic progress of the reaction was displayed on screen.

ESTIMATION OF ACID PHOSPHATASE

Serum Acid Phosphatase was estimated in control and clinical subjects. The blood was from individual cases was collected into test tubes and centrifuged to separate serum for Acid phosphatase estimation. From each test tube 0.5 ml serum was taken with a micro pipette and the Acid Phosphatase activity was estimated by the kinetic method based on U.V. absorbance at 405 nm. Acid Phosphatase content was expressed as IU/L.

It measures conversion of Phenyl Phosphate to PI and phenol, which is reacted in alkaline medium with Amino antipurine in presence of oxidizing agent Potassium Ferricyanide.

Reagents:

1. Acid Phosphatase reagent: α-napthyl phosphate 3 mm. Sodium Citrate 60 mm. pH 5.3 = 0.1.
2. L-Tartrate Reagent: Sodium L-Tartrate 2M Citric Acid 70 mm. Sodium Citrate 10 mm. pH 5.3 = 0.1
3. Acetate Buffer = 5 μ. pH = 5.0.
   Reconstitue acid phosphate dissolve with 1.0 ml of distilled water.

Procedure: To Acetate Buffer (10 μl), 1 ml of reagent was added. Test tubes were incubated at 37^{0}C for 5 mins. To it 0.5 ml serum was added, immediately it was measured at 405 nm analyser against distilled water. The reaction mixture was aspirated immediately. The instrument was programmed at 60 seconds delay to measure fall in Absorbance 405 nm. Rate of change of OD/Unit time is calculated (Δ) which was multiplied with factor. Final result as well as graphic progress of the reaction was displayed on screen.

ESTIMATION OF LACTATE DEHYDROGENASE

Serum Lactate Dehydrogenase was estimated in control and clinical subjects. The blood samples from control subjects and patients were collected into test tubes and centrifuged to separate serum for LDH estimation. From each test tube 25 µl of serum was taken with a micro pipette and the LDH content was estimated by a U.V. Spectro Photometer method. This method is based on conversion of lactate to pyruvate by NAD which is reduced to NADH. The rate of NADH formation measured 340 nm is directly proportional to serum LDH activity.

Reagents: Reconstitute reagent, contains L-lactate 75 mm, NAD 5.5 mm, buffer 80 mm, pH 9.0 $\pm$ 0.1 (30^0C) is dissolved with 10 ml distilled water.

Procedure: To LDH-L reagent added 1 ml of distilled water in series of labeled test tubes. Tubes were pre-incubated at 37 degrees centigrade for 3 mins. Subsequently 25 µl serum was added to the reagent, mixed properly and incubated at 37^0C for 1 min which was measured at 340 nm in analyser against distilled water. The reaction mixture was aspirated immediately. Machine was programmed to record Δ 340 nm at 60 second delay. Rate of change of OD/Unit time is calculated (Δ) which was multiplied with factor. Final result as well as graphic progress of the reaction was displayed on screen.