Comparison Of Platelet Count By Automated And Manual Methods, A Study And Review Of Literature In A Medical College Hospital In Kashmir

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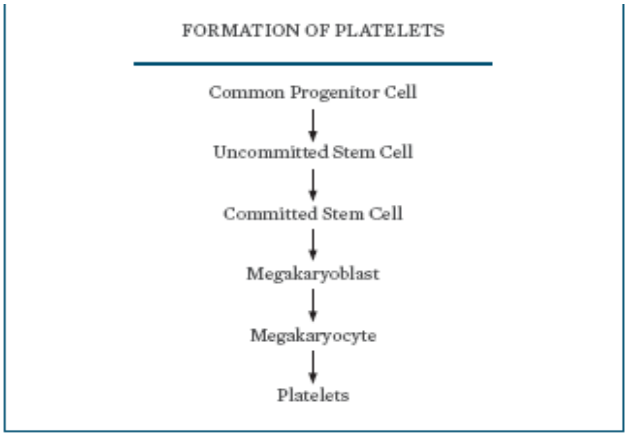
Abstract

Introduction: Estimation of platelet counts is of prime importance in clinical practice as well as in certain diseases.Automated analysers commonly used for platelet analysis may produce erroneous results in presence of particles or light scatter like fragmented red blood cells and giant platelets and platelet clumps, so alternative methods like using chamber counting and peripheral blood smear examination can be used for validation. **Aims and Objectives**: Comparison of platelet counts by three methods: 1. Automatic analyser. 2. Manual platelet count using counting chamber and 3. Traditional method , counting average platelets per ten high power fields[100X] and multiplying the same by 15000. **Methods**: Four hundred Ethylene Diamine tetra-acetic Acid (ED TA) samples were analysed over a period of six months .Statistical analysis was done by using microsoft software. Descriptive statistics like mean and percentage were used for data interpretation. Sensitivity and specificity of the manual methods was compared with that of automated analyzesr. **Results**: The mean platelet count by automated method was 1.70/µl. The mean platelet count by Neubauer chamber is 1.77/µl and mean platelet count by PBF was 1.81/µl. The P value for automated versus Neubauer chamber was 0.57 and for automated method versus PBF was 0.87 Suggesting that there was no significant variation in the platelet count between the manual methods when compared with automated analyzer. **Conclusion :**Platelet count by manual method using chamber for counting as well as by traditional method using peripheral blood smears for platelet counting are validated as alternative and reliable methods of platelet counting.

Keywords—Platelet count; automated analyser; peripheral blood smear; thrombocytes,

I. INTRODUCTION

Platelets or thrombocytes are the formed elements of blood. Platelets are small colorless, non nucleated and moderately refractive bodies, these formed elements of blood are considered to be the fragments of cytoplasm. Platelets are 2 to 4 µ in diameter and 7 to 8 cuµ in volume. Normally, platelets are of several shapes viz. Spherical or rod shaped and become oval or disc shaped when inactivated, arising from the fragmentation of megakaryocytes. Megakaryocytes are giant cells in the bone marrow, form platelets by pinching off and extruding pieces of cytoplasm.



Hemostasis, the process of stopping bleeding, is the primary function of platelets. To accomplish this, platelets contain lysosomes (chemicals capable of breaking down other substances), clotting factors, and a growth factor that stimulates healing. Traveling in the circulation, platelets join with other blood components to limit blood loss. Platelets may also help maintain the integrity of the vascular lining and stimulate proliferation of vascular smooth muscle.

Blood coagulation is a complex process. Activated by factors at the site of an injured blood vessel, platelets aggregate (collect) to form a plug, change shape, discharge their granules, and initiate the generation of thrombin, an enzyme that converts fibrinogen to fibrin. Thrombin causes them to become sticky and adhere irreversibly to each other, as well as to the break in the vessel wall. The granules attract more platelets, and thrombin begins formation of a true clot with a net of fibrin.

Platelets, besides being acute phase reactants, are also influenced by the patient`s health and nutritional status [1, 2]. Sometimes the platelets have

dumbbell shape, comma shape, cigar shape or any other unusual shape. Inactivated platelets are without processes or filopodia and the activated platelets develop processes or filopodia. Platelet is constituted by cell membrane, microtubules, and cytoplasm.

Platelets are inactive and execute their actions only when activated. Activated platelets immediately release many substances. This process is known as platelet release reaction. Platelets are responsible for formation of intrinsic prothrombin activator. This substance is responsible for the onset of blood clotting. In the blood clot, blood cells including platelets are entrapped in between the fibrin threads. Cytoplasm of platelets contains the contractile proteins, namely actin, myosin and thrombosthenin, which are responsible for clot retraction. Platelets accelerate the heamostasis. Platelets secrete 5-HT which causes the constriction of blood vessels. Due to adhesive property, the platelets seal the damage in blood vessel like capillaries. By formation of temporary plug, the platelets seal the damage in blood vessels. Platelet-derived growth factor formed in cytoplasm of platelets is useful for the repair of the endothelium and other structures of the ruptured blood vessels.

Platelet disorders occur because of pathological variation in platelet count and dysfunction of platelets. Platelet disorders are: Thrombocytopenia, thrombocytosis, thrombocythemia, Glanzmann's thrombasthenia.

Thrombocytopenia is the decrease in platelet count. It leads to thrombocytopenic purpura ; it is a disorder characterized by prolonged bleeding time. However, clotting time is normal. Characteristic feature of this disease is spontaneous bleeding under the skin from ruptured capillaries. It causes small tiny hemorrhagic spots in many areas of the body. The hemorrhagic spots under the skin are called purpuric spots (purple colour patch like appearance). That is why this disease is called purpura. Thrombocytopenia is caused due to many conditions like acute infections, acute leukemia, aplastic and pernicious anemia, chickenpox, smallpox, splenomegaly, scarlet fever, typhoid, tuberculosis, purpura, Gaucher's disease.

Thrombocytosis is the increase in platelet count. Thrombocytosis can occur due to conditions like allergic conditions, asphyxia, hemorrhage, bone fractures, surgical operations, splenectomy, rheumatic fever, and trauma.

Thrombocythemia is the condition with persistent and abnormal increase in platelet count. Thrombocythemia occurs due to many conditions like carcinoma, chronic leukemia, Hodgkin's disease.

Glanzmann's thrombasthenia is an inherited hemorrhagic disorder caused by structural or functional abnormality of platelets. It leads to *thrombasthenic purpura*. However the platelet count is normal. It is characterized by normal clotting time, normal or prolonged bleeding time but defective clot retraction.

Giulio Bizzozero, an Italian pathologist is credited with identifying platelets as an important component of blood and their essential involvement in the mechanism of blood clotting at the end of the nineteenth century [3]. It is a well known fact that an accurate and reproducible platelet counts are of utmost importance in day-to-day clinical practice aiding not only in diagnosis but also in the treatment and prognostication of a wide variety of diseases [4]. Severe thrombocytopenia can present as internal and external bleeding which maybe fatal and require emergency platelet transfusion. At the other extreme, thrombocytosis can cause thrombotic events [5]. In infections like malaria and dengue-with thrombocytopenic patients facing the potential risk of bleeding-an accurate count is imperative. Platelet count is also one of the parameters to assess the severity of dengue [6]. It is also important to prognosticate patients undergoing cytotoxic therapy for hematological malignancies. Studies have shown that platelet count is often reduced than normal in patients of Nonalcoholic fatty liver disease (NAFLD), thereby providing a supportive indicator in addition to liver biopsy in these patients. At present, platelet count is also used for prognostication of fibrosis and cirrhosis of the liver [7]. Various principles of doing automated platelet counts are impedance, optical scattering, optical fluorescence, and immunologic flow cytometry. The manual phase contrast microscopy method, although considered the gold standard was abandoned as it was time-consuming and not precise at low counts.

Immunological flow reference method also known as the RBC platelet ratio method replaced the manual phase contrast microscopy method as the international decade, which has led to greater precision in platelet counting and has resulted in its extensive use in laboratories worldwide. [8]. A few limitations of estimating platelet counts on Leishman stain smears are improper staining and stain artifacts. To overcome this, study by Uma Shankar et al [9] has shown that even the unstained peripheral smears prepared from fresh blood can be used to evaluate a platelet counts. However micro platelets are difficult to appreciate and large platelets resemble WBCs on unstained smears which must be considered [10]. A major factor contributing to the accuracy of platelet counts is the duration of sample storage post collection. The standard guidelines state that processing of hematological samples should be done within six to eight hours of collection, to avoid variations and errors in reporting the different hematological parameters [9, 11]. A study by Jain et al has shown that platelet counts done on automated analyzer s showed an unacceptable bias even within less than four hours of sample storage at 33 degree centigrade for platelet counts [12]. In the present study, all the samples were run within a span of five hours from the time of collection.

Although hematology analyzers provide reliable full blood counts based on their linearity limits, they are known to be inaccurate and poorly reproducible at enumerating platelets in severe thrombocytopenia which maybe secondary to interference from cells or materials of a similar size to platelets or due to light scatter like in the case of fragmented RBCs, microcytic RBCs, lipemic samples, debris in patients taking cytotoxic drugs or in the presence of giant platelets or platelet clumps. According to a study done by Barbara et al [8]. It was found that majority of the automated analyzers overestimated the platelet count especially when the levels were less than $200\times10^3/\mu L$. Overestimation of thrombocytopenic platelet counts may result in the substantial under transfusion of platelets in high risk patients in need of platelet transfusion resulting in significant risk of under transfusion [8] .

Therefore manual platelet estimation using various methods has been commonly used in settings of low platelet count for microscopically corroborating the auto analyzer counts. The evaluation of the peripheral blood smear till date still constitutes an indispensable tool in the evaluation of patients with hematologic disorders [13]. Manual Blood film evaluation offers added assessment of platelet size, shape, granulation, and can also be used to analyze platelet aggregation or satellitism. It is also essential for any standard of care test to be consistent, and a study carried out by Al-Hosni et al [4] demonstrated that manual platelet count estimation is reproducible in trained competent hands when a standardized methodology is used. In our study the manual methods were done independently by two observers and average values obtained were used so as to keep interobserver bias to the minimum. An average of interobserver CVs in the range of 10-25% has been reported in previous studies as against CVs of less than 3% for automated blood counts [14]. A few studies have suggested the standardization of the manual platelet count methods in relation to the microscopes used for doing the counts, the region of the smears examined on the slides and the laboratory personnel involved. Also proposals that determination of a correction factor be performed in each laboratory in order to improve the accuracy and reliability of platelet estimates [15] .

Average life span of platelets is 10 days. It varies between 8 and 11 days. Platelets are destroyed by tissue macrophage system in spleen. So, splenomegaly decreases platelet count and splenectomy increases platelet count.

The accurate platelet count estimation has an important role in diagnosis and treatment of thrombocytopenia cases. The reliability of platelet count is highly desired where the platelet transfusion is necessary. Thrombocytopenia is commonly associated with various conditions like bacterial sepsis, terminal liver disease renal failure, leukemia, malignancy, after chemotherapy etc [16, 17].

Platelet count is important diagnostic tool so it is necessary to count the platelets accurately. Before the widespread use of automated counters manual platelet count was done. The manual count is the oldest means of counting platelets and remarkably, is still used as the gold standard international reference method (18). Recently, a new immunoplatelet counting procedure has been proposed as the new international reference method. Although modern impedance counters are rapid, precise and reproducible, they tend to overestimate the platelet count in samples that contain cellular debris, e.g. thalassaemia, thrombocytopenic purpura (TTP). It is necessary to count as the transfusion threshold for platelets is now set at $10\times10^9/l$. [19,20]. Recent analyzers work on optical counting methods. [21, 22]. New methods increase the accuracy of the count as both normal and large- sized platelets are easily discriminated from other cell population.

Automated hematology analyzer:

In most of the laboratories, the hematology analyzer is utilized to count platelets in patient blood samples. Platelets are difficult to count because of their small size, marked variation in size, tendency to aggregate and overlapping of size with microcytic red cells, cellular fragments, and other debris. In hematology analyzers, this difficulty is addressed by mathematical analysis of platelet volume distribution so that it corresponds to log normal distribution. Platelets are counted by electrical impedence method in the RBC aperture, and a histogram is generated with platelet volume on X-axis and relative cell frequency on Y-axis. Particles greater than 2fl and less than 20fl are classified as platelets by the analyzer.

However, varying platelet count results may be observed with different hematology analyzer for the same blood sample, which make the comparison very difficult. The other methods for estimation of platelet count are manual counting by counting chamber, PBS examination, immunoplatelet counting and radioisotope labeling technique. [23]. Automated hematology analyzers are frequently used in clinical laboratories to assess and monitor health condition of patients.

In today's general screenings, CBC tests are performed using automated hematology analyzers. In addition to reporting RBC, PLT, and WCB counts, an analyzer also measures the oxygen-containing hemoglobin (HGB) and determines a range of other parameters such as the mean cell volume (MCV), PLT width distribution, and hematocrit (HCT), that is, the red blood cell-to-plasma ratio. Hence, an automated analyzer can provide much more information than a manual count.

Automated analyzer is adventitious in speed with efficient handling of a large number of samples, accuracy and precision in quantitative blood tests, ability to perform multiple tests on a single platform, significant reduction of labor requirements and it is

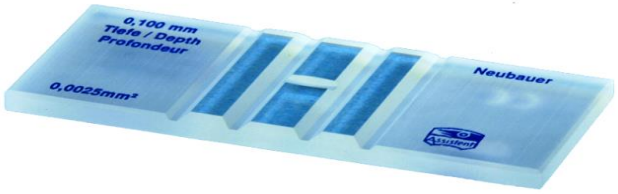
invaluable for accurate determination of red cell indices.

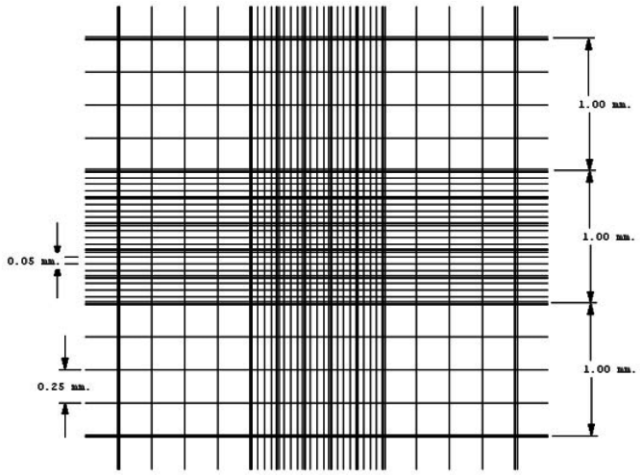
Automated analyzer have some disadvantages like:

- Comments on red cell morphology cannot be generated. Abnormal red blood cell shapes (such as fragmented cells) cannot be recognized.
- Flags: Flagging of a laboratory test result demands labor-intensive manual examination of a blood smear.
- Erroneously increased or decreased results due to interfering factors
- Expensive with high running costs.

Haemocytometer:

A haemocytometer consists of a counting chamber, a cover glass for the counting chamber and the diluting pipettes. The haemocytometer or Neubauer chamber consists of a thick rectangular glass slide with an`H' shaped trough, forming two counting areas. Beyond the two vertical arms of the trough are two raised shoulders which support the specially made thick, optically flat cover glass. Space between the cover glass and surface of the counting area [depth] is exactly 0.1mm. Each counting area is 3×3 mm. This produces a total counting volume of 0.9cu.mm. When the ruled area is viewed under the 10x objective of the microscope it shows 5 squares. Each having 1mm² area. This 1mm² sections in the four corners are divided into 16 equal squares. The square in the centre of the ruled area is divided into 25 equal squares. In turn each of these 1/25mm² squares are further divided into 16 portions.





Technical Errors

Errors in bood collection.

- Prolonged, tight application of a tourniquet leads to venous stasis and false elevation of cell count.
- Excessive squeezing of finger puncture results in dilution of blood with tissue fluid.
- Inadequate mixing of blood with anticoagulant leads to formation of clots in blood sample and falsely low count.
- Non-mixing of anticoagulated venous blood imme-diately before testing will cause falsely low count.

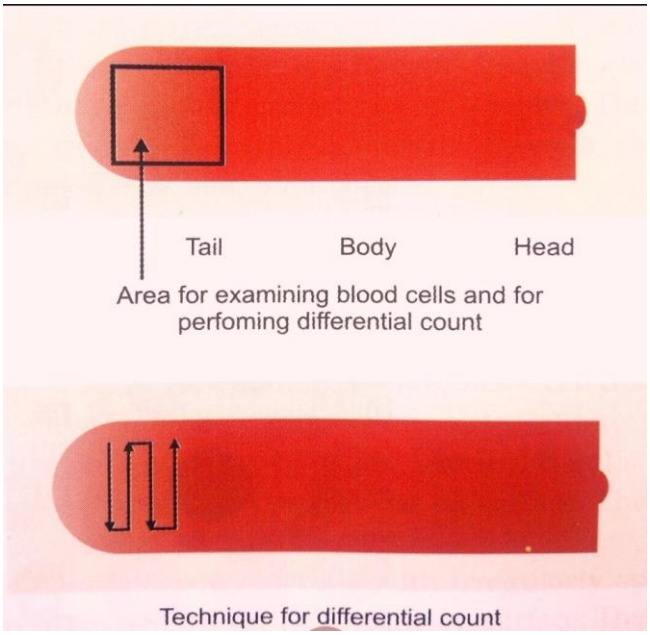
Errors in pipetting

- Use of wet, chipped, or dirty pipettes
- Use of improperly calibrated pipettes
- Not wiping blood adherent to outside of pipette
- Using bulb pipettes that are difficult to calibrate and which break off easily.

Errors in filling of chamber

- Incorrect filling of chamber, spillage into moats
- Not allowing 2 minutes for cells to settle.
- Drying of edges of preparation
- Not using specified coverglass
- Chamber containing dirt or air bubble.

Peripheral blood smear:



Manual methods are time consuming, subjective and tedious with high levels of imprecision [10]. Automated hematology analyzers though rapid in giving results, at times give erroneous values in the presence of giant platelets, platelet clumps, fragmented and microcytic red blood cells. [24, 25, 26]

It is a standard protocol in most hematology laboratories, that abnormal platelet values generated by cell counter be followed by a manual examination of Leishman staind peripheral blood smear [27, 28, 29]

PBS or peripheral blood smear is highly informative hematological tool for RBC`S, WBC`S, platelets morphology and counting. PBS screening is commonly used for disease diagnosis and its progress and therapeutic response. A peripheral blood smear has three parts: head, body and tail. A blood smear should be examined in an orderly manner. The best morphological details of PBS are seen in the area where red cells are just touching one another. The platelet count results obtained by this method of PBS examination had no statistically significant difference when compared with hematology analyzer platelet count results for same blood sample [30, 31]. Microscopic examination of PBS is performed by preparing, staining and examine a thin film of blood. It provides valuable information on the status of blood cells and presence of parasitic elements. The blood film is the only permanent record of hematological investigations. Therefore a well made well stained smear is essential. The film must be prepared in a technically correct manner so that the cells are evenly distributed throughout the smear. An ideal blood film should be smooth and not interrupted by ridges and holes. It should cover ½ to ¾ of the area of the slide. It should be thickest at the origin or the head and gradually thin out forming a tail without defined border at the end. The thickness and the length of the film will depend on the angle of the spreader, the size of the drop of blood and speed at which the spreader is pushed forward. It is essential to use a clean slide. A glass slide cleaned with alcohol and wiped dry gives the best result. A spreader e.g. margin free slides with ground glass edges, should be used to make the film. After preparation the blood smear should be stained as soon as possible.

In the present study, we analysed the platelet count results obtained by hematology cell analyser, Neubauer counting chamber and Peripheral Blood Smear, examination in randomly selected blood samples.

Review Of Literature

Wallace H Coulter discovered the Coulter Principle in the late **1940s** (though a patent was not awarded until October 20, 1953). Coulter was influenced by the atomic bombs dropped on Hiroshima and Nagasaki. These events motivated Coulter to simplify and improve blood cell analysis so that large populations could be screened rapidly, as would be necessary in the event of a nuclear war. Partial funding of the project came from a grant award from the Office of Naval Research. [32, 33]

Webb Dl, et al., (2004) studied the platelet count visually in a peripheral blood smear and compared it with an automated machine platelet count. 35 peripheral blood smears were made from blood specimens counted on an automated blood cell machine. Ten and 25 high-power fields were microscopically averaged and then multiplied by 15,000 and 20,000 to arrive at a platelet count in 1,000 per micro liter. Multiplying by 15,000 gives a platelet count reasonably close to automated machine counts in 1000 per microliter.

Anitha K,et al., (2013) conducted the study to compare the platelet estimation by peripheral smear method and automated method and the result of this study suggest that platelet estimation by peripheral smear method is a reliable, rapid, easy and economic, it can be done even in rural setup for early diagnosis of thrombocytopenia in pregnancy, as it is equivalent to the automated method.

Parvaiz. A. Shah, et al., (2013) studied the role of mean platelet volume in the pathogenisis, severity and outcome of isochemic stroke. The prospective study comprised of 100 patients each of isochemic stroke with equal number of age and sex matched control group. High mean platelet volume and low platelet count was found in the study group with in comparison to the control group was statistically significant. Observations showed that, mean platelet volume bears an inverse relationship to immediate outcome from ischemic stroke independent of stroke subtype.

Carlo L. Balduini, et al., (2014) put together all data of subjects enrolled in the three population based studies concluding the age related changes in platelet count were actually very large: platelet count in old age was reduced by 35% in men and by 25% in women with respect to early infancy. Most of the reduction occurred in childhood and in old age, with only minor changes in adulthood. Thus, there is no longer any doubt that age is a major determinant of platelet count in healthy people.

Zainab Mohammedi Golwala, et al., (2016) Studied forty cases and controls. Platelet count, PCT and the ratios of MPV/platelet count, MPV/PCT, PDW/platelet count, PDW/PCT and MPV × PDW/platelet count × PCT were significantly different among children who survived compared to those who died. On multiple regression analysis the ratio of MPV/PCT, PDW/ platelet count and MPV/platelet count were risk factors for mortality with an odd ratio of 4.31, 3.86, 3.45 respectively which is 95% of CI. In 67% of patients who died MPV/PCT ratio was above 41.8 and PDW/platelet count was above 3.86. In 65% of patients who died MPV/platelet count was above 3.45.

Sajad Geelani, et al., (2017) studied the variation in platelet count between automated analyzer and manual platelet counting. It was found that platelet count by manual method is higher as compared to the automated method in the laboratory. Higher platelet count by manual method is because of large platelet size which analyzers are not able to count.

Balakrishnan A, et al., (2018) received 250 samples in EDTA tubes for platelet counts during the study period. 121 were female and 129 were males.

Mean age of the patients was 45.8 years. Study has shown that manual methods are reliable to validate the automated counts in routine practice under standard conditions. In addition essential that pathology laboratory personals and clinicians who rely on platelet counts for various scenarios in day to day practice understand the limitation of instrumentation in use and the measurement uncertainty of automated platelet counters.

Vyankatesh T. Anchinmane, et al., studied 100 randomly collected blood samples in EDTA anticoagulant vacutainer tubes. Each blood sample was processed for platelet count estimation with automated hematology analyzer and Leishman's stained PBS examination. The statistical analysis was done by using Pearson's correlation test to access the agreement between both the methods. Study concluded that platelet count estimation by PBS method is reliable and statistically significant when compared to hematology analyzer method.

Aims and objectives:

Platelet count is one of the critical parameters in patient care, at times being a decisive factor for diagnosis and treatment. There are several methods of platelet count used in hematology laboratory. These methods are:

- Manual counting method.
- Automated hematology analyzer counting method.
- Platelet count estimation by PBS method etc.

The present study was undertaken to estimate platelet counts by automated hematology analyzer and correlate them with results obtained from PBS and Neubauer chamber.

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Materials and methods;

The investigation on blood platelet count on the random Kashmiri population was carried out in pathology and hematology department of SKIMS-Medical college Hospital (Bemina). The study involved following steps for meeting various objectives of study:

1. Collection of blood sample in EDTA coated vials.
2. Processing of blood samples for platelet count estimation with automated analyzer, PBS and Neubauer chamber.
3. Statistical analysis.

1. Collection:

A total of 400 blood samples from random *Kashmiri* population were used to conduct the study. All the venous blood samples were collected in EDTA vials. Each blood sample was properly mixed and the vials were properly labeled.

2. Processing:

2.1. Hematology automated analyzes;

The platelet count was obtained by processing blood samples in fully automatic hematology analyzer (COULTER LH 750). It is a quantitative, automated hematology analyzer and leukocytes differential counter for invitro diagnostic use in clinical laboratories. The coulter H750 hematology analyzer provides automated reticulocyte analysis and enumeration of nucleated red blood cells (NRBC'S) as well as an automated method for enumeration of RBC'S and WBC'S in body fluid.

The purpose of the LH700 series is to separate the normal patient, with all normal system generated parameters, from the patient who needs additional studies of any of these parameters. These studies might include further measurements of cell size and platelet distribution, manual WBC differential or any other definitive test that helps diagnose the patient's condition.

PARAMETERS:

WBC white blood cell or leukocyte count

RBC Red blood cell or erythrocyte count

Hgb Hemoglobin concentration

Hct Hematocrit (relative volume of erythrocytes)

MCV Mean corpusculas (erythrocyte) volume

MCH Mean corpuscular (erythrocyte) heamoglobin

MCHC Mean corpuscular(erythrocyte) heamoglobin concentration

RDW red cell (erythrocyte volume) distribution width

Plt platelet or thrombocyte count

MPV Mean platelet (thrombocytes) volume

LY% Lymphocyte percent

MO% Monocyte percent

NE% Neutrophil percent

EO% Eosinophil percent

BA% Basophil percent

LY# Lymphocyte number

MO# Monocyte number

NE# Neutrophil number

EO# Eeosinophil number

BA# Basophil number

NRBC% Nucleated red blood percent

NRBC# Nucleated red blood cell number

RET% Reticulocyte percent

RET# Reticulocyte number

*HLR% High light scatter reticulocytes %

*HLR# High light scatter reticulocyte%

IRF Immature reticulocyte fraction

MRV Mean reticulocyte volume

*MSCV Mean sphered cell volume

*PCT Plateletcrit

*PDWS Platelet distribution width

Automated hematology analyzers work on different principles ;

- Electrical impedance
- Light scatter
- Fluorescence
- Light absorption
- Electrical conductivity.

Most analyzers are based on a combination of different principles.

2.1.1 Electrical impedance

This is the classic and time-tested technology for counting cellular elements of blood. As this method of cell counting was first developed by Coulter Electronics, it is also called as Coulter principle. Two electrodes placed in isotonic solutions are separated by a glass tube having a small aperture. A vacuum is applied and as a cell passes through the aperture, flow of current is impeded and a voltage pulse is generated. The requisite condition for cell counting by this method is high dilution of sample so that minimal numbers of cells pass through the aperture at one point of time. There are two electrodes on either side of the aperture as the solution in which the cells are suspended is an electrolyte solution; an electric current is generated between the two electrodes. When cell passes through this narrow aperture across which a current is flowing, change in electrical resistance (I. e. Momentary interruption of electrical current between the two electrodes) occurs. A small pulse is generated due to a temporary increase in impedance. This pulse is amplified, measured, and counted. The height of the pulse is proportional to cell volume. The width of the pulse corresponds with the time required for the cell to traverse the aperture. Cells that do not pass through the center of the aperture generate a distorted pulse that is not representative of the cell volume. Some analyzers use hydrodynamic focusing to force the cells through the central path so that all cells take the same path for volume measurement. An anticoagulated whole blood sample is aspirated into the system, divided into two portions, and mixed with a diluent. One dilution is passed to the red cell aperture bath (for red cell and platelet counting), and the other is delivered to the WBC aperture bath (where a reagent is added for lysis of red cells and release hemoglobin this portion is used for leukocyte counting followed by estimation of hemoglobin). Particles between 2-20 Fl are counted as platelets, while those between 36-360 fl are counted as red cells. Hemoglobin is estimated by light transmission at 535 nm.

2.1.2 *Light scatter*

Each cell flows in a single line through a flow cell. A laser device is focused on the flow cell as the laser light beam strikes a cell it is scattered in various directions. One detector captures the forward scatter light (forward angle light scatter or FALS) that is proportional to cell size and a second detector captures side scatter (SS) light (90 °) that corresponds to the nuclear complexity and granularity of cytoplasm. This simultaneous measurement of light scattered in two directions is used for distinguishing between granulocytes, lymphocytes, and monocytes.

2.1.3. *Fluorescence*

Cellular fluorescence is used to measure RNA (reticulocyte), DNA (nucleated red cells), and cell surface antigens.

2.1.4. *Light absorption*

Concentration of hemoglobin is measured by absorption spectrophotometry, after conversion of hemoglobin to cyanmethemoglobin or some other compound. In some analyzers, peroxidase cytochemistry is used to classify leukocytes; the peroxidase activity is determined by absorbance.

2.1.5. *Electrical conductivity*

Some analyzers use conductivity of high frequency current to determine physical and chemical composition of leucocytes for their classification.

2.2 Peripheral blood smear

➢ **Principle**

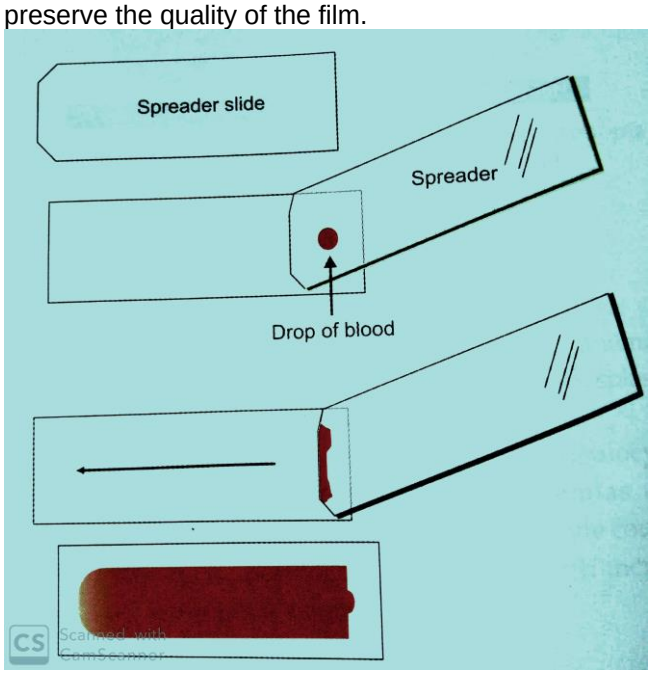
The polychromic staining solution (wright, Leishman, Giemsa) contain methylene blue and eosin. These basic and acidic dyes induce multiple colors when applied to cells. Methanol acts as fixative and also as a solvent. The fixative does not allow any further change in the cells and make them adhere to the glass slide.

➢ **Procedure**

A thin smear is prepared by spreading a small drop of blood evenly on a slide.

2.2.1 Making a film

- Take a clean, dry slide.
- Transfer a small drop of blood near the edge of slide.
- Place the spreader slide at an angle of 30 degree.Pull back the spreader until it touches the drop of blood. Let the blood run along the edge of the spreader.
- Push the spreader forward to the end of this slide with a smooth movement .Dry the blood smear at room temperature. Adequate drying is essential to preserve the quality of the film.



2.2.2 Identification marking

- By using glass marker or lead pencil, write the identification number on the slide.

2.2.3 Fixing the smear

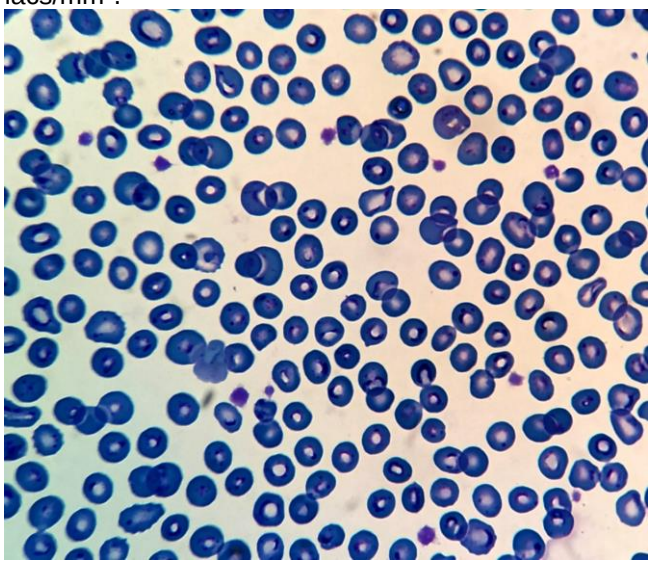
- The blood smear is fixed with methanol for 2-3 minutes to prevent distoration of cells

2.2.4 Staining the film

- Covered the smear with the staining solution (Leshman stain) by adding 10-15 drops on the smear. Wait for 1 minute.
- Add equal number of the drops of buffer solution. Mix the reaction mixture adequately by blowing on it through a pipette. Wait for 10 minute.
- Watch the smear by using tap water.
- Stand the slide in a draining rack or on the laboratory counter to dry.

2.2.5 Examination of film

- First examined the stained smear under the low power. Three zones will appear Head, body and tail.
- The portion slightly before the tail-end where the red cells begin to overlap is chosen.
- Then a drop of immersion oil is placed on the smear and examines the film by moving from one field to next.
- Count the platelets in at least 10 oil immersion fields. The average number of platelet is multiplied by 15×10^4 and the platelet count is expressed as lacs/mm^3.



2.3 Neubauer chamber

2.3.1 Principle

Whole blood sample is mixed with a diluent (1% Ammounium oxlate) in which red cells are lysed while the leukocyte, platelets and reticulocytes remain intact. The dilution for platelet count is 1:200. The sample was incubated for some time and mounted on haemocytometer. The cells were allowed to settle and then counted in a specific area of the haemocytometer chamber under the microscope. The number of platelet was calculated per microlitre.

2.3.2Material

- Blood sample (EDTA)
- Neubauer counting chamber
- Adjustable pipettes
- 1% Ammonium oxalate solution for dialution
- Test tubes
- Test tube rack
- Glass capillary tubes
- Petridish with wet filter paper
- Microscope

2.3.3 Method

- Test tubes were labeled and 3.98ml of 1% ammonium oxalate is taken in each test tube.
- To this add 20µl of well mixed anticoagulated venous blood and mix thoroughly. Dilution of blood is 1:200.
- Diluted samples were mixed by inversion.
- Mirrored surface of the counting chamber and glass cover slip is cleaned gently.
- Place coverslip over the heamocytometer.
- Capilary tube full of well mixed diluted sample was filled.
- Capillary tube was touched to the edge of the loading groove on one side of the counting chamber and we allowed the diluted sample to fill the chamber
- The counting chamber was placed in a petridish in a moist filter paper for 15 minutes to allow the platelets to settle
- Bottom the chamber was wiped carefully to remove excess moisture from the moist petriplate and place chamber on the microscopic stage
- Using the 10x objective, we focused on the engraved counting area to look for the central 1mm square
- Then we change to 40x objective
- The platelets will appear like highly refractile particles
- Count platelets in all 25 small squares the area covered by 25 squares is equivalent to 1 sq.mm

2.3.4Calculation

The formula used for calculating the cell count was

$$\text{Platelets/mm}^3 = \frac{\text{number of platelet counted} \times \text{dilution}}{\text{area counted} \times \text{depth of fluid}}$$

Where;

Dilution = 200

Area =1mm^2

Depth = 0.1mm

$$\text{Platelets/mm}^3 = \frac{\text{Number of platelets} \times 200}{0.1}$$

= Number of platelets ×2000

3 Statistical analysis

Statistical analysis was done by using Microsoft office software. Descriptive statistics like mean and percentage were used for data interpretation. Sensitivity and specificity of the manual methods was compared with the autoanalyzer.

Result and discussion

In present study, 400 samples of blood were collected from the random Kashmiri population and were processed by automated analyzer (Backman coulter LH 750) and manual platelet counting (Neubauer chamber) and Peripheral blood examination .

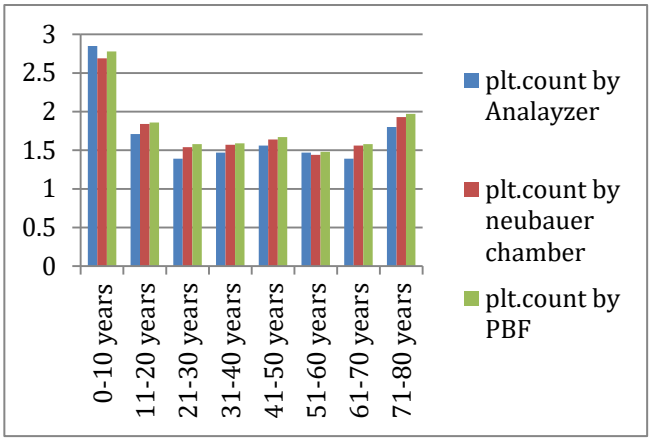
A total of 400 samples were received in EDTA tubes for platelet counts during the study period, 167 were males and 233 were females.

The result demonstrated variation in platelet count between automated analyzer and manual platelet counting.

Age wise variation of platelet count using automated platelet count analyzer, Neubauer and PBF is given in table 1.

Age group Years	*Frequency of patients*	*Male*	*Female*	*Plt. count by Analayzer*	*Plt. count by Neubauer chamber*	*Plt .count by PBF*
0-10	9	4	5	2.85	2.71	2.78
11-20	53	27	26	1.71	1.84	1.86
21-30	135	36	99	1.39	1.54	1.58
31-40	88	34	54	1.47	1.57	1.59
41-50	60	33	27	1.56	1.64	1.67
51-60	38	23	15	1.47	1.44	1.48
61-70	15	9	6	1.39	1.56	1.58
71-80	2	1	1	1.80	1.93	1.97
TOTAL	**400**	**167**	**233**	**13.64**	**14.37**	**14.51**

Table 1: Age wise variation of platelet count.



Method	Total cases	Mean	Std. deviation	P value
Automated method	400	1.705	0.485	0.57
Neubauer chamber	400	1.776	0.403	

Table 2: Comparison between automated analyzer and Neubauer chamber.

Method	Total cases	Mean	Std. deviation	P value
Automated method	400	1.705	0.485	0.87
PBF	400	1.813	0.422	

Table 3: Comparison between automated analyzer and PBF.

The mean platelet count by Automated method was 1.70/µl. The mean platelet count by Neubauer chamber is 1.77/µl and mean platelet count by PBF was 1.81/µl. The P value for automated versus Neubauer chamber was 0.57 and for automated method versus PBF was 0.87 Suggesting that there was no significant variation in the platelet count between the manual methods when compared with automated analyzer.

Discussion:

The precise, accurate and reliable assessment of platelet count is required to avoid unnecessary platelet transfusion in the treatment of severe thrombocytopenia patients. The accuracy is also needed after platelet transfusion treatment to check the therapeutic response.

The platelets circulate in the blood as small disc and are derived from megakaryocytes in the bone marrow. Megakaryocyte constitutes <1% of myeloid cells in the bone marrow. One megakaryocyte can give rise to one thousand to three thousand platelets. The platelets are about 3µm in diameter and are non-nucleated. The life span of normal platelet is about seven to twelve days and is destroyed by splenic macrophages. In wet preparation, platelet appears as colorless, discoid or elliptical refractile bodies. In Leishman`s stained PBS, platelets appears as light blue colored, round, oval or rod shaped structures. Platelet are multifunctional and plays key role in hemostasis, thrombosis and wound repair. The normal range of platelets count in healthy human being is 15×10^4 to 40×10^4 platelets perµl. The thrombocytopenia is one of the critical conditions where patients platelet count decreases below the normal range. [34,35]

The platelet count is commonly done in laboratories by evaluation of PBS, Neubauers chamber counting or by automated hematology analyzer. The platelet counting is more difficult as compared to RBCS count and WBC count. Until recently, the Brecher and Cronkite platelet counting method described in 1950, was consider as reference method while comparing with platelet counting by semi-automated and automated method. These manual phase contrast microscopic method was considered as gold standard which had significant limitation in view of imprecision. This method results are highly variable and depends upon individual training. Few researchers mentioned that the risk of error estimation is upto 10% to 20% for this manual platelet counting method. [35]

In the year2001, a joint task force of ISLH and ICSH recommended a new immunological based reference method for platelet counting. This method utilizes monoclonal antibodies to platelet surface antigens conjugated to suitable flurophore. This method permits the possible implementation of new reference method for calibration of hematology analyzer. [36]

The platelet count with hematology analyzer is usually precise. In hematology analyzers, the principle of particle impedence is utilized which was first described by Wallace H. Coulter in 1954. The hematology analyzer platelet count accuracy is compromised while processing blood samples with low platelet counts or with blood samples with abnormal platelets morphology like giant platelets or blood samples having presence of non platelet particle like RBC, WBC fragments. The accuracy of platelet count is also compromised in hematology analyzer due to inadequate calibrations and lack of adequate quality control material.[37, 38] Despite of advances in hematology automation, manual counting methods has its own importance in hematology laboratories for validating results of other methods for platelet counting. Till date, even the accurate and best quality hematology analyzer also cannot replace the manual counting evaluation. [39]

Web et al and Bajpai et al had reported slightly better results with 15.0×10^4 platelets per µl as platelet multiplication factor instead of 20 $\times10^4$ platelets per µl [40, 30]. The PBS platelet count estimation method gives approximate platelet count and not the exact on and is the drawback of this method. However PBS method precisely comments on adequacy of platelet count in

patient blood samples and hence this method has upper hand over other methods in diagnosing thrombocytopenia cases and avoiding unnecessary platelet transfusion.

Neubauer chamber inspite of being the best manual counting technique, an error still occurs due to random distribution of the cells in the chamber. This results in variation in cell number in different areas of the same size. This distribution follows Poisson`s law. More number of cells should be counted to reduce this error.

The present study has shown that manual methods are reliable to validate the automated counts in routine practice under standard conditions and can be used as alternative methods for platelet estimation as well as the standard methods in rural clinical set ups where automatic analysers are a remote possibility.

In addition, it is essential that pathology laboratory personals and clinicians who rely on platelet counts for various scenarios in day to day practice understand the limitations of the instrumentation in use and the measurement uncertainty of automated platelet counters.

However, limitation of the study are a small sample size and that reproducibility of the manual methods in case of thrombocytopenia, thrombocytosis and normal ranges have not been studied independently.

Conclusion:

The study highlights the differences in platelet count in the population by using automated and manual counting methods. During analysis it was found that platelet count by manual methods is a little higher as compared to the automated method, the possible reason for this could be the large sized platelets (Giant platelets with size ranging from 10-20µm) which the analyzers are not able to count.

The study tries to validate the manual methods for use in circumstances where in a fully automated hematology analyzer is not available as is usually seen in in primary healthcare centers or urban health centre. This also reaffirms that manual methods may also be used to validate the results shown by analyzer.

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